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# PATHOGENIC
# MICROÖRGANISMS

## A PRACTICAL MANUAL FOR STUDENTS, PHYSICIANS AND HEALTH OFFICERS

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# PREFACE TO THE SEVENTH EDITION.

THE first edition of this book was called *Bacteriology in Medicine and Surgery*. It was written to make available for others the practical knowledge which had been acquired in the work of the bacteriological laboratories of the city of New York, and was intended more for medical practitioners than for medical students or laboratory workers. When the second edition had been exhausted the improvement in methods of cultivating and studying the protozoa had reached a point rendering it advantageous to include the animal as well as the vegetable germs. This was done and the title of the third edition was altered to conform with the text, which had been broadened to give in outline practically the whole field of pathogenic microörganisms. At the same time the subjects were treated in a more comprehensive manner, so as to make it a suitable text-book for medical students.

In the fifth edition the material was rearranged in order to bring more closely together all of the pathogenic organisms. Under the arrangement Part I deals with the general characteristics and methods of study of all the microörganisms considered (moulds, yeasts, bacteria and protozoa). Part II includes the study of the individual pathogenic microörganisms and their near relatives. Part III presents certain practical aspects of the subject under the title Applied Microbiology. In the sixth edition the practical application of serums and vaccines was transferred also to Part III. The nine plates included in this edition have been arranged and drawn especially for this book.

Owing to recent advances in the knowledge of microbiology and the opportunity afforded by a new edition, we have again added considerable new material and rewritten several parts of the book. The whole subject of immunity has been rewritten. The chapter on Media has been practically rewritten by Dr. B. v. H. Anthony and utilizes the recent work on hydrogen-ion concentration. The sections on streptococci, on yeasts and on influenza bacilli have been extensively revised. The information gained during the influenza epidemic upon bacteria pathogenic for the respiratory tract and during the last part of the war

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with preventive measures against typhoid fever, paratyphoid fevers and wound infections due to anaërobes has been added. The chapter on Complement Fixation was revised by M. A. Wilson and W. C. Noble. The index and much of the proofreading was done by N. Eldridge. We are further indebted to our associates in the laboratory for aid in many different ways.

W. H. P.
A. W. W.
C. K.

NEW YORK, 1920.

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# PATHOGENIC MICROÖRGANISMS.

# PART I.

## PRINCIPLES OF MICROBIOLOGY.[1]

### CHAPTER I.

### INTRODUCTORY HISTORICAL SKETCH.

ALTHOUGH most of the more important discoveries in microbiology which place it on the footing of a science are of comparatively recent date, the foundations of its study were laid over two centuries ago. From that time the history of microörganisms has been intimately associated with that of medicine. Indeed, it is only through the investigations into the life history of these minute forms that our present knowledge of the etiology, course and prevention of the infectious diseases has been acquired. The prominent position which the study of microörganisms already holds toward medicine is, moreover, daily increasing in importance. Original discoveries are constantly adding to our knowledge of germ diseases, and the outlook is favorable for eventually obtaining, through serums, through attenuated cultures, or through the toxic substances produced by microörganisms themselves, means for immunizing against, if not of curing, an increased number of the specific infections. Even at present, bacterial products and protective serums are used successfully as preventive or curative agents in several of the most prevalent infectious diseases. Our knowledge concerning other microörganisms has enabled us largely to limit their dissemination and so to prevent disease. An acquaintance, therefore, with the main facts concerning these microörganisms is most necessary to the education of the modern physician.

The vast majority of the known microörganisms which cause disease belong to the closely related groups of lowest plants and animals, *i. e.*, the bacteria, the moulds, the yeasts and the protozoa. A few of the pathogenic metazoa (some of the parasitic worms) in some of their stages are also microscopic; therefore microscopic methods of study are also applicable to them; but since they are fully presented in works on clinical microscopy they are not given here.

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[1] The correct form of this word is under discussion. According to derivation—*microbe* + *logia*—the better spelling would be *microbology*, but the form given above is the one accepted at present in the dictionaries.

Before entering into a detailed consideration of the subject it may be interesting and instructive to review very briefly a few of the important steps which led to the development of the science, and upon which its foundation rests, in which we shall see that the results obtained were gained only through long and laborious research and after many obstacles were met and overcome by accurate observation and experiment.

Probably the first authentic observations of living microscopic organisms of which there is any record are those of Kircher, in 1659. This original investigator demonstrated the presence in putrid meat, milk, vinegar, cheese, etc., of "minute living worms," but did not describe their form or character.

Not long after this, in 1675, Leeuwenhoeck observed in rainwater, putrid infusions, and in his own and other saliva and diarrheal evacuations living, motile "animalcula" of most minute dimensions, which he described and illustrated by drawings. Leeuwenhoeck practised the art of lens grinding, in which he eventually became so proficient that he perfected a lens superior to any magnifying glass obtainable at that day, and with which he was enabled to see objects very much smaller than had ever been seen before. "With the greatest astonishment," he writes, "I observed distributed everywhere through the material which I was examining animalcules of the most microscopic size, which moved themselves about very energetically." The work of this observer is conspicuous for its purely objective character and absence of speculation; and his descriptions and illustrations are done with remarkable clearness and accuracy, considering the imperfect optical instruments at his command. It was not until many years later, however, that any attempt was made to define the characters of these minute organisms and to classify them systematically.

At that time all of the microscopic organisms seen were classed together as little animals. Indeed, all of the microörganisms first described at any length were probably protozoa, and only after further improvement of the lenses and a more minute study of the organisms were bacterial forms gradually recognized as a separate class.

The first ideas of the structure of the protozoa were drawn from analogy. The early observers thought that each tiny organism possessed an internal structure made up of organs and tissues similar to those in metazoa. They could not conceive of motion without articulation, tendons and muscles; nor of food absorption without an alimentary tract, and they were so impressed with the ideas of what they thought they ought to see that they were convinced that they really saw many of the complicated structures possessed by metazoa. For example, the contractile vacuole, a characteristic pulsating vesicle of the protozoa, discovered by Joblot, in 1754, was thought by many to be lungs, other vacuoles were said to be stomachs, the mouths were often seen and the rest of the alimentary tract was supplied from the imagination; the red pigment spots of many forms were interpreted as true eyes, etc. There were many opponents to these views, however, and the idea of the cell being the unit of structure, which was advanced by Schleiden, in 1838, helped determine the fact that protozoa were single cells with no definite organic structure.

With the publications of Dujardin (1835–1841) a correct idea of the structural simplicity of the microörganisms gained ground. But for some time after the controversy regarding the simple nature of protozoa was strenuously carried on. It is a most instructive bit of history in research work, showing how the lack of minute observation, the exercise of a too vivid imagination, and the close reasoning from analogy may lead one astray, while the proper use of these functions may bring out the truth.

From the earliest investigations into the life history and properties of germs, microörganisms have been thought to play an important part in the causation of infectious diseases. Shortly after the first investigations into this subject the opinion was advanced that puerperal fever, measles, smallpox, typhus, pleurisy, epilepsy, gout and many other diseases were due to contagion. In fact, so widespread became the belief in a causal relation of these minute organisms to disease that it soon amounted to a veritable craze, and all forms and kinds of diseases were said to be produced in this way upon no other foundation than that these organisms had been found in the mouth and intestinal contents of men and animals and in water.

Among those who were specially conspicuous at this time for their advanced views on the germ theory of infectious diseases was Marcus Antonius Plenciz, a physician of Vienna. This acute observer, who published his views in 1762, maintained that not only were all infectious diseases caused by microörganisms, but the infective material could be nothing else than a living organism. On these grounds he endeavored to explain the variations in the period of incubation of the different infectious diseases. He also insisted that there were special germs for each infectious disease by which the specific disease was produced. Plenciz believed, moreover, that these organisms were capable of multiplication in the body, and suggested the possibility of their being conveyed from place to place through the air.

These views, it is true, were largely speculative, and rested upon insufficient experiment, but they were so plausible, and the arguments put forward in their support were so logical and convincing, that they continued to gain ground, in spite of considerable opposition and ridicule, and in many instances the conclusions reached have since been proved to be correct. The mode of infection, its unlimited development among large numbers of individuals, and gradual spread over wide areas—the incubation, course of, and resulting immunity in recovery from infectious diseases—all pointed to a living organism as the probable cause.

Among other distinguished men of the day whose observations exerted a most powerful influence upon the doctrine of infection, may be mentioned Henle. His writings (*Pathological Investigations*, 1840, and *Text-book of Rational Pathology*, 1853) in which he described the relation of microörganisms to infectious diseases and defined the character and action of bacteria upon certain phases and symptoms of these affections, are remarkable for their clearness and precision.

**Origin of Microörganisms.**—But, meanwhile, the question which most interested these investigators into the cause of infectious diseases was: Whence are these microörganisms derived which were supposed to produce them? Were they the result of spontaneous generation due to vegetative changes in the substances in which the organisms were found, or were they reproduced from similar preëxisting organisms—the so-called vitalistic theory? This question is intimately connected with the investigations into the origin and nature of fermentation and putrefaction.

Spallanzani, in 1769, demonstrated that if putrescible infusions of organic matter were placed in hermetically sealed flasks and then boiled, the liquids were sterilized; neither were living organisms found in the solutions, nor did the infusions decompose; they remained unchanged for an indefinite period.

An objection was raised by the believers in spontaneous generation that in excluding the oxygen of the air by hermetically sealing the flasks, the essential condition for the development of fermentation which required free admission of this gas was interfered with. This objection was met by Schulze, in 1836, by causing the air admitted to the boiled decomposable liquids to pass through strong sulphuric acid. Air thus robbed of its living organisms did not produce decomposition.

Schwann, in 1839, obtained similar results in another way: He deprived of microörganisms the air admitted to his boiled liquids by passing it through a tube which was heated to a temperature high enough to destroy germs. To this investigator is also due the credit of having discovered the specific cause—the yeast plant, or *Saccharomyces cerevisiæ*—of alcoholic fermentation, the process by which sugar is decomposed into alcohol and carbonic acid.

Again it was objected to these experiments that the heating of the air had perhaps brought about some chemical change which hindered the production of fermentation. Schroeder and von Dusch, in 1854, then showed that by a simple process of filtration, which has since proved of inestimable value in bacteriological work, the air can be mechanically freed from germs. By placing in the mouth of the flask containing the boiled solutions a loose plug of cotton, through which the air could freely circulate, it was found that all suspended microorganisms could be excluded, and that air passed through such a filter, whether hot or cold, did not cause fermentation of boiled infusions.

Similar results were obtained by Hoffmann, in 1860, and by Chevreul and Pasteur, in 1861, without a cotton filter, by drawing out the neck of the flask to a fine tube and turning it downward, leaving the mouth open. In this case the force of gravity prevents the suspended bacteria from ascending, as there is no current of air to carry them upward through the tube into the flask containing the boiled infusion.

Although in the large majority of cases it was found possible to keep boiled organic liquids sterile in flasks to which the oxygen of the air had free access, the question of spontaneous generation still remained unsettled, inasmuch as occasionally, even under the most careful precautions, decomposition did occur in such boiled liquids.

This fact was explained by Pasteur, in 1860, by experiments showing that the temperature of boiling water was not sufficient to destroy all living organisms, and that, especially in alkaline liquids, a higher temperature was required to insure sterilization. He showed, however, that at a temperature of 110° to 112° C., which he obtained by boiling under a pressure of one and one-half atmospheres, all living organisms were invariably killed. Pasteur, at a later date (1865), demonstrated the fact that the organisms which resist boiling temperature are, in fact, reproductive bodies, which are now known as *spores*.

These facts have since been practically confirmed on a large scale in the preservation of food by the process of sterilization. Indeed there is scarcely any biological problem which has been so satisfactorily solved or in which such uniform results have been obtained; but all through the experiments of the earlier investigators irregularities were constantly appearing.

When and how life began no one is yet able to say. That spontaneous generation may even be taking place now under unknown conditions is conceivable, but all such ideas are purely hypothetical and there is no evidence that under present conditions any of the known microörganisms have originated in any way except from a previous similar cell.

Stimulated by the establishment of the fact, through Pasteur's investigations, that fermentation and putrefaction are due to the action of living organisms reproduced from similar preëxisting forms, and that each form of fermentation is due to a special microörganism, the study of the causal relation of microörganisms to disease was taken up with renewed vigor. Reference has already been made to the opinions and hypotheses of the earlier observers as to the microbic origin of infectious diseases. The first positive grounds, however, for this doctrine, founded upon actual experiment, were the investigations into the cause of certain infectious diseases in insects and plants. Thus, Bassi, in 1837, demonstrated that a fatal infectious malady of the silkworm—*pébrine*—was due to a parasitic protozoön. Pasteur later devoted several years' study to an exhaustive investigation into the same subject; and in like manner Tulasse and Kühne showed that certain specific affections in grains, in the potato, etc., were due to the invasion of parasites.

Very soon after this it was demonstrated that microörganisms were probably the cause of certain infectious diseases in man and the higher animals. Davaine, a famous French physician, has the honor of having first demonstrated the causal relation of a microörganism to a specific infectious disease in man and animals. The anthrax bacillus was discovered in the blood of animals dying from this disease by Pollender in 1849 and by Davaine in 1850; but it was not until 1863 that the last-named observer demonstrated by inoculation experiments that the bacillus is the cause of anthrax.

The next discoveries were those relating to wounds and the infections to which they are liable. Rindfleisch, in 1866, and Waldeyer and von Recklinghausen, in 1871, were the first to draw attention to the minute organisms occurring in the pyemic processes resulting from infected wounds, and occasionally following typhoid fever. Further investigations were made by Billroth, Fehleisen and others, in erysipelatous inflammations secondary to injury, who agreed that in these conditions microörganisms could almost always be detected in the lymph channels of the subcutaneous tissues.

The brilliant results obtained by Lister, in 1863–1870, in the antiseptic treatment of wounds to prevent or inhibit the action of infective organisms, exerted a powerful influence on the doctrine of bacterial infection, causing it to be recognized far and wide and gradually lessening the number of its opponents. Lister's methods were suggested to him by Pasteur's investigations on putrefaction.

In 1877, Weigert and Ehrlich recommended the use of the anilin dyes as staining agents and thus made possible a more exact microscopic examination of microörganisms in cover-glass preparations.

In the year 1880 Pasteur published his discovery of the bacillus of fowl cholera and his investigations upon the attenuation of the virus of anthrax and of fowl cholera, and upon protective inoculation with attenuated germs against these diseases. In the meantime he showed that pure cultures might be obtained by the dilution method.

Laveran, in the same year, announced the discovery of parasitic bodies, the first pathogenic protozoa described in humans, in the blood of persons sick with malarial fever, and thus stimulated investigations upon the immensely important pathogenic unicellular animal parasites.

In 1881, Koch published his fundamental researches upon pathogenic bacteria. He introduced solid culture media (agar had already been used by Frau Hess) and the "plate method" for obtaining pure cultures, and showed how different organisms could be isolated, cultivated independently, and, by inoculation of pure cultures into susceptible animals, could be made, in many cases, to reproduce the specific disease of which they were the cause. To him more than to any other are due the methods which have enabled us to prove absolutely, in a broad sense, the permanence of bacterial varieties. It was in the course of this work that the Abbé system of substage condensing apparatus was first used in bacteriology. Koch's postulates, which were at this time formulated, helped to further careful researches and a critical examination of the proofs before any microbe was accepted as the causal organism of a disease. These are: (1) A specific organism must always be associated with a disease, (2) when isolated in pure culture (3) and inoculated into a healthy susceptible animal it must always produce the disease and (4) should be obtained again in pure culture. These were long accepted as the only proof of the causal relationship of an organism to the disease. Later it was learned that immunological reactions added greatly to our knowledge of the specificity of microbes in disease, and can afford proof without demonstrating all of Koch's laws.

In 1882 Pasteur published his first communication upon rabies. The method of treatment devised by him is still in general use. A little later came the investigations of Löffler and Roux upon the diphtheria bacillus and its toxins, and that of Kitasato upon tetanus. These researches paved the way for Behring's work on diphtheria antitoxin, which in its turn stimulated investigation upon the whole subject of immunity. The number of investigators rapidly increased as the importance of the earlier fundamental discoveries became apparent. Additions to the science of microbiology have been made from many sides, and the practical application of the facts learned from these investigations is steadily increasing. Recent additions have been made chiefly toward the side of the prevention of disease. The results obtained during the war just ended constitute a striking demonstration of how much can now be accomplished, even under adverse conditions, in preventing many diseases that were earlier great scourges. For instance, typhoid, paratyphoid, smallpox and cholera were made practically *nil* by protective vaccination; tetanus was controlled by protective inoculations of antiserum; gas gangrene was prevented by special disinfectant; plague was avoided by rat extermination; typhus and trench fever were prevented by delousing; malaria was controlled by mechanical measures, by quininization and by draining. Many of these results were obtained under the stress of war. It remains to be seen whether similar and even greater efforts will be made to prevent disease in the future.

For a more detailed account of the history of the development of bacteriology and protozoölogy in medicine see the following references:

Calkins: Protozoölogy, 1909, Lea & Febiger, New York and Philadelphia.

Löffler, Fried: Vorlesungen über die geschichtliche Entwickelung der Lehre von den Bakterien, Leipzig, F. C. W. Vogel, 1887, with bibliography.

Valleri, Radot: Life of Pasteur.

Armand-Delille, P. F.: Role of Pasteurian Methods in Prophylaxis of Infectious Diseases, Jour. Am. Med. Assn., 1918, lxxi, 1809.

# CHAPTER II.

## CLASSIFICATION AND GENERAL CHARACTERISTICS OF MICROÖRGANISMS.

In general the lowest forms of life are microscopic. Among these microscopic organisms are many which have in common the ability to produce disease in the higher animals and plants. Some of these disease-producing or pathogenic microörganisms are plants, others are animals, while some are difficult to classify. Only those that are of direct importance to man are considered in this work.

### CLASSIFICATION OF MICROÖRGANISMS.

The classification of microörganisms is still in the transition stage. This is due chiefly to the difficulties encountered in studying the individual morphological characteristics of such minute bodies and in determining their limits of variation.

There is no one distinctive characteristic known which separates the lowest plants from the lowest animals. While the lowest microorganisms of all—the bacteria—are usually classed as plants, their structure is so simple and their biological characteristics are so varied that their relationship to the vegetable kingdom is not clear-cut. In their possession of more or less rigid plasmolysible bodies, in the tendency of many to grow in filaments, in their division apparently by simple fission, and in the ability of some to use simple elements as food, they resemble plants; while in the motility of many, the nonpossession by all of chlorophyl, and in the necessity of many for complex food, they resemble animals.

There is a similar difficulty in definitely classifying many members of the other groups of closely related microörganisms, namely, the protozoa, the yeasts and molds, and it has been suggested that under the name *Protista* a third kingdom be formed consisting of all of these lowest microörganisms.

As a rule, in the more minute organisms genera are based upon morphological characteristics, and species upon biochemical, physiological and pathogenic properties. This is due to the fact that the morphology of varieties may vary extremely under different conditions and that it may give no indication whatever of the relation of microörganisms to disease and fermentation—the chief characteristics of importance to human beings.

The properties of bacteria, for example, which are fairly constant under uniform conditions and which have been more or less used in systems of classification, are those of spore and capsule formation, motility (flagella formation), reaction to staining reagents; relation to temperature, to oxygen and to other food material, and, finally, their relation to fermentation and disease. But any one of these properties, in many groups of microörganisms, such as the coli and the streptococcus groups, under certain conditions may so vary that, taking it as a basis for classification, an organism might be dropped from the group with which it has been classified and be placed in an entirely different group. Thus, the power to produce spores or flagella may be held in abeyance for a time or may be totally lost; the relations to oxygen may be gradually altered, so that an anaërobic bacterium may grow in the presence of oxygen or the contrary may be the case; parasitic organisms may be so cultivated in the test-tube as to become for the time saprophytic varieties, and those which when taken from their natural habitat have only faint traces of the power to grow in the living body may be made to acquire pathogenic properties when they are made to develop in a series of animals of the same species whose resistance has been overcome by reducing their vitality through poisons or other means.

**Permanence of Species.**—It is true, however, that under favorable and uniform conditions microörganisms retain most of their characteristics for an indefinite time. While we cannot believe that the multitude of varieties which now exist have always existed, and we may theorize as to the part played by the fluctuating variations just mentioned in producing permanent species, the facts observed in the few years during which bacteria have been studied is that most pathogenic species as observed in disease have remained practically unaltered. The diphtheria bacilli are the same today as when Löffler discovered them in 1884, and the disease itself is evidently the same as history shows it to have been before the time of Christ. The same permanence of disease type is true for tuberculosis, smallpox, hydrophobia, leprosy, etc. Under practically unchanged conditions, therefore, such as exist in the bodies of men, most germs which have once become established as parasites continue to reproduce new generations which retain their peculiar (specific) characteristics. It is true that among the countless organisms developed some fail to hold the parasitic characteristics. These either continue as saprophytes or cease to exist. That new pathogenic disease varieties are coming into existence from time to time is, of course, a possibility, but not a certainty. Such *true* mutations have been from time to time reported, notably by Penfold[1] for the coli group and by Jordan[2] and by Barber[3] for the dysentery group, but whether or not the changed characteristics may be considered species characteristics cannot at present be decided.

The work of Rosenow,[4] of Irons[5] and others on elective localization of streptococci shows how marked may be the adaptability of certain bacteria to different conditions of growth.

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[1] Jour. Hyg., March, 1911, xi, 30-67.

[2] Proc. Nat. Acad. of Sci., 1915, i, 160.

[3] Phila. Jour. Sc., 1913, iii, 539.

[4] Jour. Am. Med. Assn., 1915, lxv, 1687.

[5] Jour. Inf. Dis., 1916, xviii, 315.

Our lack of more definite knowledge in regard to the significance of these changes, as we have said, is the chief cause of many unsatisfactory results from attempts at classification.

At present a systematic effort is being made by the Society of American Bacteriologists to formulate rules for the adoption of a classification that will be accepted by all.[1] Until such a universally accepted classification can be determined it seems wiser to note only such a broad, simple grouping as the one given below.

### WORKING CLASSIFICATION OF PATHOGENIC MICROÖRGANISMS IN OUTLINE.

| Kingdom. | Subkingdom. | Classes in which pathogenic species occur. | Genera in which chief pathogenic species occur. |
|---|---|---|---|
| Plants (Fungi) | Moulds (Hyphomycetes) | Mycomycetes | Aspergillus, Penicillium. |
| | | Phycomycetes | Mucor. |
| | | Unclassified (Fungi imperfecti) | Microspora, Trichophyta, Sporotricha, Achoria, Coccidiodes. |
| | Yeasts (Blastomycetes) | Oïdia<br>Monilia<br>Torula<br>Saccharomyces | Oïdium, Monilia, Torula, Saccharomyces. |
| | Bacteria (Schizomycetes) | Cocci (Coccaceæ) | Micrococcus, Diplococcus, Streptococcus, Tetracoccus. |
| | | Bacilli (Bacteriaceæ) | Bacillus (Bacterium). |
| | | Spirilla (Spirillaceæ) | Spirillum [Spirocheta, Treponema]. |
| | | Higher bacteria (Trichobacteria) | Cladothrix, Nocardia (Streptothrix). |
| Animals (Protozoa) | Flagellates (Mastogophora) | Flagellata | Cercomonas, Leptomonas, Herpetomonas, Trypanosoma, Leishmania, Trichomonas, Lamblia [Spirocheta, Treponema]. |
| | Amebæ (Sarcodina) | Rhizopoda | Entameba. |
| | Sporozoa | Telosporidia, Neosporidia | Eimeria, Hemogregarinida, Proteosoma, Hemameba, Babesia, Rhinosporidium, Myxobolus, Nosema, Sarcocystis, etc. |
| | Ciliates | Ciliata | Balantidium. |

Unclassified: Ultramicroscopic organisms.

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[1] Preliminary Report. Jour. Bacteriol., 1917, ii, 505.

### EXPLANATION OF PLATE I.

Partly schematic. Rearranged and drawn by Williams from the indicated authors.

FIG. 1. Aspergillus glaucus. Fruiting hyphæ growing from mycelium: *A*, conidiophore; *B*, sterigma; *C*, conidia; *D*, beginning perithecium; *E*, conidiophore bearing spores; *F*, perithecium containing rudiments in section; *G*, ascus containing eight spores (De Bary).

FIG. 2. Penicillium, showing formation of conidia, *A*.

FIG. 3.—Mucor mucedo: *A*, sporangium containing spores; *B*, spores liberated; *C*, chlamydospores; *D*, *E*, *F*, stages in the formation of a zygospore.

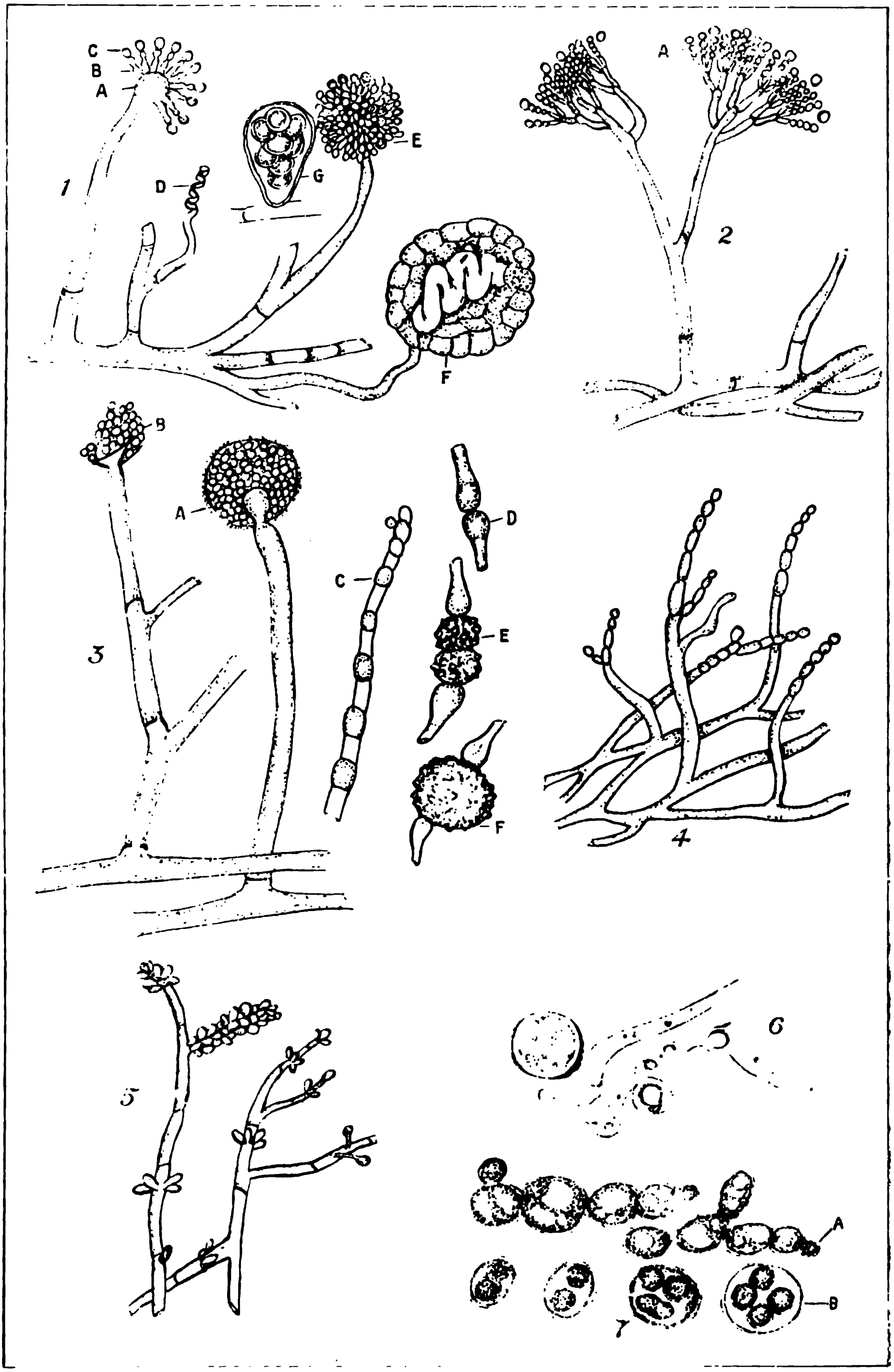
FIG. 4.—Oïdium lactis.

FIG. 5.—Sporotrichum schenkii, showing formation of whorled spores on branched mycelium.

FIG. 6.—Yeast from human infection in culture showing mycelium-like growth.

FIG. 7.—Saccharomyces cerevisiæ (Hansen): *A*, budding; *B*, spore formation.

# PLATE I



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| | Sporozoa | Telosporidia, Neosporidia | Eimeria, Hemogregarinida, Proteosoma, Hemameba, Babesia, Rhinosporidium, Myxobolus, Nosema, Sarcocystis, etc. |
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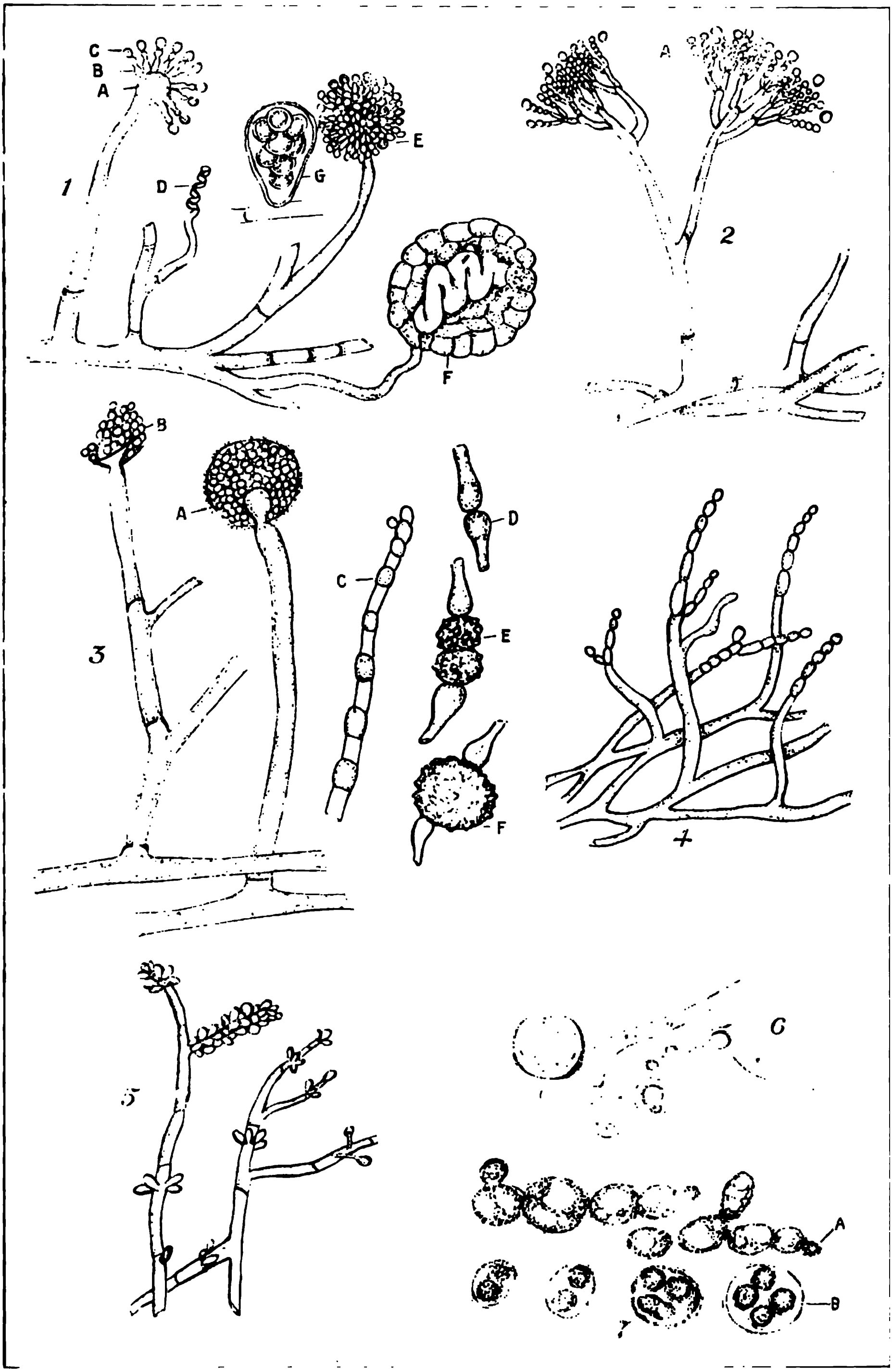
FIG. 7.—Saccharomyces cerevisiæ (Hansen): *A*, budding; *B*, spore formation.

# PLATE I



**Nomenclature.**—It is well to call attention to the fact that in naming species, especially among the bacteria, the binomial law of nomenclature has been frequently violated. Such names as Bacillus coli communis should not be accepted; the name Bacillus coli or Bacillus communis is sufficient as well as correct.

## GENERAL CHARACTERISTICS OF EACH GROUP OF MICROÖRGANISMS.

**Introduction.**—The knowledge gained from the fact that each variety of microbe possible of cultivation may grow in distinctive ways upon so-called artificial culture media has been an immense aid in studying the characteristics of the more minute forms, for the individual cell of most varieties is so minute that even the highest magnification we have may show little if any morphological difference between organisms which produce distinctly different diseases, or between a pathogenic and a non-pathogenic form. There are, however, certain morphological and biological characteristics of the individual cell in the different groups, which are pronounced, and we therefore study these before going on to the study of cultures, that is, of microörganisms in masses.

Morphological descriptions should always be accompanied by a definite statement of the habitat—if from a culture, age of the growth, the medium from which it was obtained, and the temperature at which it was developed.

## MOULDS (HYPHOMYCETES, EUMYCETES).

The moulds (see Plate I) are closely related to the higher bacteria. Like them they grow in branching filaments or threads forming a mycelium, but these filaments or hyphæ, as they are called, are more definite than those of the trichobacteria, and they are multicellular; that is, the filaments are septate, in some varieties always (mycomycetes) and in others when forming spores (phycomycetes). These two groups of moulds have other differential characteristics, particularly in their fruiting organs.

The *mycomycetes* are divided into two groups: (1) Ascomycetes, which form a spore sac, containing a definite number of spores, a multiple of two, usually eight (ascospores); (2) basidiomycetes, which form a definite spore-bearing cell called the basidium or conidiophore, from which four (usually) conidia or sterigmata protrude (mushrooms, etc.). The sexual fruiting organs are varied.

Yeasts produce a definite sporangium containing a definite number of spores (two or a multiple of two), therefore they are closely related to the ascomycetes.

The *phycomycetes* are characterized by the formation of *no definite* basidium or ascus. The asexual fruiting organs are formed in three different ways:

1. Ends of hyphæ swell and are shut off by septum, forming *sporangium* in which (*a*) swarm spores develop. These become free by rupture of sporangium wall, swim about, and then form a new plant; (*b*) spores form a cell wall while in sporangium.

2. Spores produced directly by constriction at end of certain hyphæ. Such spores are called conidia and the hyphæ conidiophores; each conidium is able to develop into a new plant.

3. Spores produced by separation of hyphæ into short-walled segments or oïdia. When the spores are thick-walled they are called chlamydospores.

Sexual reproduction occurs in one of two ways:

1. The ends of two similar hyphæ become attached, then they segment from the rest of the hyphæ and become surrounded by a thick wall. The partition in the center dissolves and the protoplasm mixes. The resulting body is called a zygospore. It produces a new plant. The members of this group are called zygomycetes.

2. The swollen ends of two dissimilar hyphæ, one large (female) and one smaller (male), become attached, fuse, segment and become surrounded by a thick wall. The body is called an oöspore. The plants are called oömycetes.

*Fungi Imperfecti.*—The moulds that are not definitely classified are divided into two groups:

1. Known forms which do not fit in the above group.

2. Not fully known forms. To this group belong most of the moulds causing human disease.

## YEASTS (BLASTOMYCETES).

These microörganisms which are called blastomycetes because of their frequent and marked method of reproduction by means of budding, have been for many centuries of the greatest importance in brewing and baking. They are not uncommonly present in the air and in cultures made from the throat. Certain experiments have shown that some varieties when injected are capable of producing tumor-like growths. Certain varieties are pathogenic for mice, and since 1894 there have been reported a number of cases of human infection from yeasts.

The position which the yeasts occupy in systematic biology has not, thus far, been accurately determined. Forms classed by some authors with the yeasts are placed by others with the Fungi imperfecti of the moulds. The chief differential characteristics between these intermediate groups and the true yeasts, or saccharomyces, may be tabulated tentatively as follows:

| | Genus. | Endospores. | Budding. | Mycelium. | Gas formation. |
|---|---|---|---|---|---|
| Blastomycetes | Saccharomyces | + | + | − | + |
| Blastomycetes<br>Fungi imperfecti | Torula | − | + | \| | − |
| | Monilia | − | + | ± | + |
| | Oidia | − | + | + | − |
| | Coccidiodes | + | − | + | − |

The most important property of yeasts, though one not possessed by all to the same degree, is that of producing alcoholic fermentation. The enzyme causing this action is called zymase. In brewing we distinguish between the yeasts that can be employed practically, "culture yeasts," and those which often act as disturbing factors, so-called "wild" yeasts.

Two other enzymes produced by yeasts have been much studied, namely, invertase which changes saccharose into dextrose and levulose, and maltase which changes one molecule of maltose into two molecules of dextrose. (See also p. 62.)

The shape of most of the culture yeasts is oval or elliptic (Plate I, Fig. 7). Round or globular forms are more often met with among the wild species which usually excite only a slight degree of fermentation. But sausage-shaped and thread forms are also met with. Pathogenic forms may also be round.

The individual yeast cells are strongly refractive, so that under the microscope at times they have almost the luster of fat droplets. This is important because in examining fresh tissues the yeast cells may be hard to distinguish from fat droplets, often requiring the aid of certain reagents for their identification.

The size of the individual yeast cells varies enormously, even in those of the same species or the same culture. In old colonies individuals may be found no larger than cocci, $0.5\mu$ to $1\mu$ in diameter, while in other colonies, especially on the surface of a liquefied medium, giant yeast cells are found often attaining a diameter of $40\mu$ or more. In spite of these wide fluctuations, however, the various species are characterized by a fairly definite average in size and form. Each cell contains a more or less definite nucleus, which is demonstrated by the usual chromogenic stains.

During the process of budding the nucleus of the cell moves toward the margin, where it divides. At this point the limiting membrane of the cell ruptures or usually a hernia-like protrusion develops which has the appearance of a button attached to the cell. The daughter-cell so formed rapidly increases in size and gradually assumes the shape and size of the mother-cell.

A fact of the utmost importance for the propagation of the blastomycetes and continuation of the species is the formation of spores which may take place either by the changing of special cells as a whole into single spores or by the dividing of the nuclear material of a cell into several fragments, each of which becomes the center of a new cell lying within the original cell (Plate I, Fig. 7). These new cells possess a firm membrane, a cell nucleus and a little dense protoplasm. The number of these endospores developed in the yeast cells varies, but is constant for a given species. As a rule, one cell does not produce more than four *endogenous* spores, but species have been observed, *e. g.*, *Schizosaccharomyces octosporus* (Beijerinck), in which eight spores are found. Guilliermond has described conjugation in yeasts before the formation of spores.

Yeasts stain readily in the young state with ordinary dyes. They are Gram-positive. Older forms stain very irregularly. They are cultivated—sometimes with difficulty directly from tissue—on ordinary media, best on media made slightly acid (see chapter on Media). Some pathogenic varieties grow best at blood heat. Most varieties grow easily at room temperature.

The vitality of yeasts is truly enormous. Hansen as well as Lindner were able to obtain a growth from cultures twelve years old. Busse succeeded in getting a luxuriant growth from a dry potato culture seven and a half years old, which was almost as hard as bone.

## BACTERIA.

**Definition.**—*Bacteria* may be defined as extremely minute, simple, unicellular microörganisms, which reproduce themselves under suitable conditions with exceeding rapidity, usually by transverse division, and grow without the aid of chlorophyl. They have no morphological nucleus, but contain nuclear material which is generally diffused throughout the cell body in the form of larger or smaller granules.

**Natural Habitat.**—Bacteria are found all over the known world. Wherever there is sufficient moisture, one form or another will find other conditions adequate for multiplication. Thus, we meet with bacterial life between 0° and 75° C. Some live only in the tissues of men, others in lower animals, a small number may grow in both man and lower animals, others still grow only in plants, but by far the greater number live in dead organic matter. For some free oxygen is necessary to life; for others it is a poison.

**Morphology** (see Plates II and III).—The form and dimensions of bacterial cells at their stage of complete development must be distinguished from those which they possess just after or just before they

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EXPLANATION OF PLATE II.

FIG. 1.—Illustrating cocci single or in irregular groups (micrococcus, staphylococcus), micrococcus from air. × 1000.

FIG. 2.—Illustrating cocci in twos—diplococcus pneumoniæ from peritoneal exudate of rabbit. × 1000.

FIG. 3.—Illustrating cocci in chains—Streptococcus pyogenes. × 1000.

FIG. 4.—Illustrating cocci in fours—Micrococcus tetragenus from spleen of mouse. × 1000.

FIG. 5.—Illustrating cocci in packets—Sarcina lutea from air. × 1000.

FIG. 6.—Illustrating large single bacilli: Bacillus subtilis (hay bacillus). × 1000.

FIG. 7.—Illustrating small bacilli; mostly in twos—Bacillus hoffmanni from human throat. × 800. (Park.)

FIG. 8.—Illustrating bacilli in chains—anthrax bacillus from spleen of mouse. × 500.

FIG. 9.—Illustrating bacilli in bunches—typhoid bacillus from human spleen. × 500.

FIG. 10.—Illustrating bacilli in threads—anthrax bacilli from blood of frog.

FIG. 11.—Spirillum undula, showing flagella. × 1000.

FIG. 12.—Cholera spirilla, gelatin culture. × 1000.

FIG. 13.—Large spirilla in chains from water. × 1000.

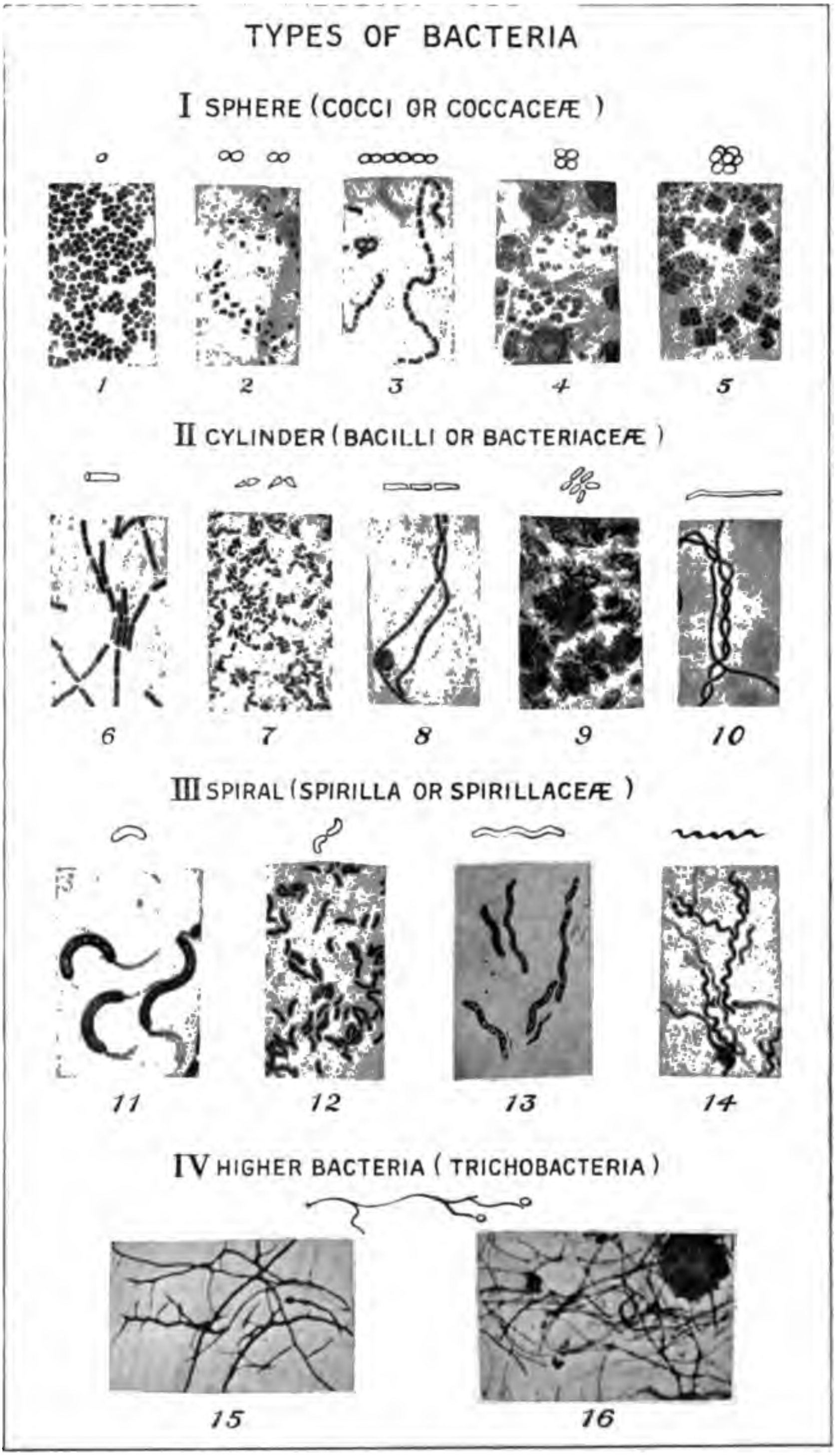
FIG. 14.—Smaller spirilla in chains—Spirillum rubrum. × 1000.

FIG. 15.—Streptothrix candida—broth culture (Zettnow, from Kolle and Wassermann).

FIG. 16.—Streptothrix hominis from sputum (Zettnow, from Kolle and Wassermann).

Unless otherwise indicated, the photographs are from Fränkel and Pfeiffer.

# PLATE II


TYPES OF BACTERIA
I SPHERE (COCCI OR COCCACEÆ)
1
2
3
4
5
II CYLINDER (BACILLI OR BACTERIACEÆ)
6
7
8
9
10
III SPIRAL (SPIRILLA OR SPIRILLACEÆ)
11
12
13
14
IV HIGHER BACTERIA (TRICHOBACTERIA)
15
16

have divided. As a spherical cell develops preparatory to its division into two cells it becomes elongated and appears as a short oval rod; at the moment of its division, on the contrary, the transverse diameter of each of its two halves is greater than their long diameter. A short rod becomes in the same way, at the moment of its division, two cells, the long diameter of each of which may be even a trifle less than its short diameter, and thus they appear on superficial examination as spheres.

**Size.**—The dimensions of the adult individual vary greatly in the different species as well as in members of the same species. The largest bacillus recorded is 50μ[1] to 60μ long and 4μ to 5μ wide (B. bütschlii, see Fig. 7). One of the smallest forms known (B. influenzæ) has an average size of 0.5μ x 0.2μ.

Some pathogenic organisms are so small (ultramicroscopic, see p. 71, also chapter on Filtrable Viruses) as to be invisible with any magnification which we now possess. We know of their existence either by the fact that they may be cultivated on artificial media, producing appearances of mass growth, and that such cultures when inoculated into susceptible animals cause the characteristic disease, or by the fact that the filtrates after passing through the pores of the finest filters are infectious.

**Shape.**—The *basic forms* of the single bacterial cells are threefold—the sphere, the rod, and the segment of a spiral. Although under different conditions the type form of any one species may vary considerably, yet these three main divisions under similar conditions are constant, and, so far as we know, it is never possible, by any means, to bring about changes in the organisms that will result in the permanent conversion of the morphology of the members of one group into that of another—that is, micrococci always, under suitable conditions, produce micrococci, bacilli produce bacilli, and spirilla produce spirilla.

As bacteria multiply, the cells produced from the parent-cell have a greater or less tendency to remain attached. This is on account of the slimy envelope which is more or less developed in all bacteria. In some varieties this tendency is extremely slight, in others it is marked. This union may appear simply as an aggregation of separate bacteria or so close that the group appears as a single cell. According to the method of the cell division and the tenacity with which the cells hold together, there are different groupings of bacteria, which aid us in their differentiation and identification. Thus, in cocci we get the bacterial cell dividing into one, two, or three planes (Plate II, Figs, 1 to 5), while in bacilli and spirilla the division is generally in only one plane (Plate II, Figs. 6 to 9).

1. Spherical Form, or Coccus.—The size varies from about 0.15μ as minimum diameter to 3μ as maximum; the average size of the pathogenic cocci is 0.8μ. The single elements are, at the moment of their complete development, so far as we can determine, practically spherical;

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[1] A μ, or micromillimeter, is $\frac{1}{25000}$ of an inch.

but when seen in the process of multiplication through division the form is seldom that of a true sphere. Here we have elongated or lancet-shaped forms, as frequently seen in the diplococcus of pneumonia, or the opposite, as in the diplococcus of gonorrhea, where the cocci appear to be flattened against one another. Those cells which divide in one direction only and remain attached are found in pairs (diplococci) or in shorter or longer chains (streptococci). Those which divide in two directions, one at right angles to the other, form groups of four (tetrads). Those which divide in three directions and cling together form packets in cubes (sarcinæ). Those which divide in any axis form irregularly shaped, grape-like bunches (staphylococci).

2. Rod Form, or Bacillus.—The type of this group is the cylinder. The length of the fully developed cell is always greater than its breadth. The size of the cells of different varieties varies enormously: from a length of 30$\mu$ and a breadth of 4$\mu$ to a length of 0.2$\mu$ and a breadth of 0.1$\mu$. The largest bacilli met with in disease do not, however, usually develop over 3$\mu$ x 1$\mu$, while the average is 2$\mu$ x 0.5$\mu$. Bacilli are roughly classed, according to their form as slender when the ratio of the long to the transverse diameter is from 1 to 4 to 1 to 10, and as thick when the proportion of the long to the short diameter is approximately 1 to 2.

The characteristic form of the bacillus has a straight axis, with uniform thickness throughout, and flat ends; but there are many exceptions to this typical form. Thus, frequently certain bacilli have rounded ends,

## EXPLANATION OF PLATE III.

Partly schematic (Williams). Stained by Giemsa except where otherwise indicated.

Figs. 1 to 4.—Anthrax bacilli at different stages of development.

Fig. 1.—Shows chromatin-staining masses (red), in plastin-staining cells (light blue).

Fig. 2.—Shows same organism after one hour on fresh medium, chromatic substance distributed throughout medium and whole organism taking deeper, more homogeneous stain.

Fig. 3.—Organism showing two kinds of granules according to staining powers.

Fig. 4.—Same organism placed on fresh medium extrudes some granules (waste products) and redissolves others (chromatin granules).

Figs. 5 to 9.—Similar granules in diphtheria bacilli at various stages of development.

Fig. 10.—Metachromatic granules in Bacillus pyocyaneus, Neisser stain.

Fig. 11.—Metachromatic granules in sarcinæ, Neisser stain.

Fig. 12.—Metachromatic granules in Bacillus influenza, Giemsa's stain.

Fig. 13.—Metachromatic granules in gonococci, Giemsa's stain.

Fig. 14.—Pneumococcus, capsule stained by Hiss method.

Fig. 15.—Pneumococcus mucosus (Streptococcus mucosus) by Welch stain for capsule.

Fig. 16.—Rhinoscleroma bacillus by Hiss stain for capsules.

Fig. 17.—Plasmolysis in cholera bacillus showing capsule.

Figs 18 to 21 inclusive.—Types of flagella by Löffler stain.

Fig. 18.—Monotricha, cholera vibrio.

Fig. 19.—Amphitricha, water bacillus.

Fig. 20.—Lophotricha, spirillum undula.

Fig. 21.—Peritricha, typhoid bacillus.

Fig. 22.—Formation and end germination of spores in anthrax.

Fig. 23.—Lateral germination of spore in Bacillus subtilis.

Fig. 24.—Central germination of spores in Bacillus alvei (Wilson).

Fig. 25.—Type of spores: *A*, central; *B*, eccentric; *C*, end.

Fig. 26.—Diphtheria bacilli in old cultures, Giemsa's stain.

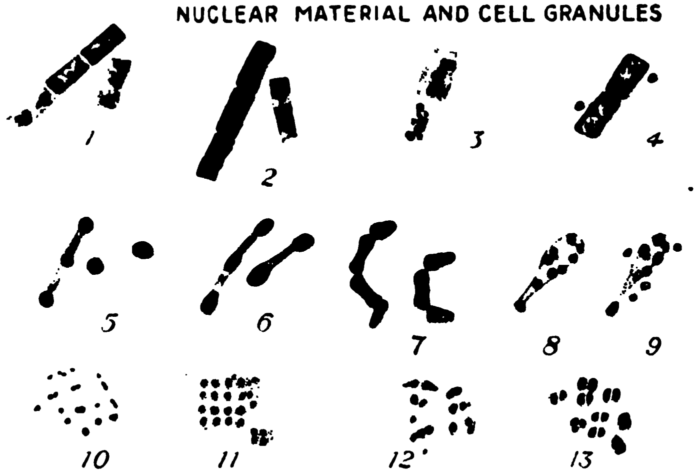
Fig. 27.—Plague bacilli in old cultures, stained by methylene blue.

Fig. 28.—Influenza bacilli in old cultures, Giemsa's stain.

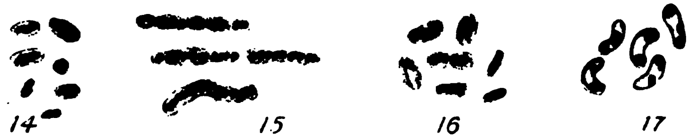
# PLATE III

## STRUCTURE OF BACTERIA

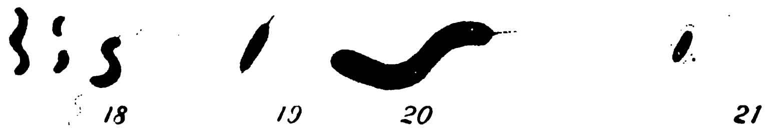
### NUCLEAR MATERIAL AND CELL GRANULES



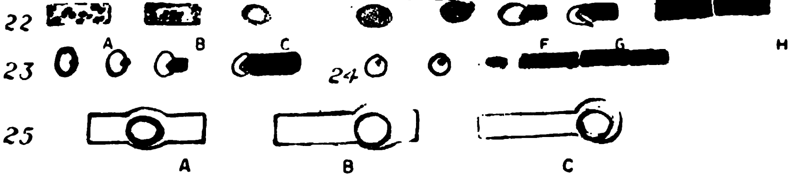
### CAPSULES AND MEMBRANES



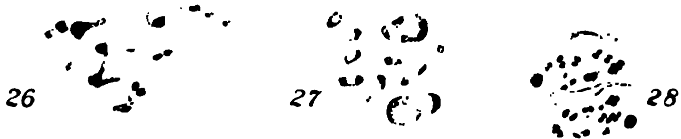
### FLAGELLA



### SPORES



### IRREGULAR FORMS IN OLD CULTURES (INVOLUTION FORMS)



A. W. WILLIAMS, DEL.

many of the more slender forms have the long axis, slightly bent; some few species, as for example the diphtheria bacilli, invariably produce many cells whose thickness is very unequal at different portions. Spore formation also causes an irregularity of the cell outline.

The bacilli, except when they develop from spores or granules, divide only in the plane perpendicular to their long axis. A classification, therefore, of bacilli according to their manner of grouping is much simpler than in the case of the cocci. We may thus have bacilli as isolated cells, as pairs (diplobacilli), or as longer or shorter chains (streptobacilli).

3. Spiral Form, or Vibrio.—The members of the third morphological group are spiral in shape, or only segments of a spiral. Here, too, we have large and small, slender and thick spirals. The twisting of the long axis, which here lies in two planes, is the chief characteristic of this group of bacteria. Under normal conditions the twisting is uniform throughout the entire length of the cell. The vibrio, like the bacilli, divide only in one direction. A single cell, a pair, or the union of two or more elements may thus present the appearance of a short segment of a spiral or a comma-shaped form, an S-shaped form, or a complete spiral or corkscrew-like form.

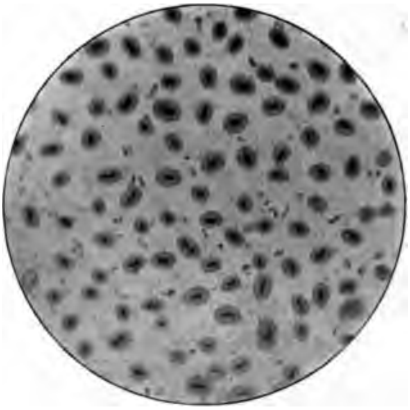


Fig. 1.—Bacillus pneumoniæ (Bacillus mucosus) stained for capsule by Huntoon's method. × about 900. (Huntoon.)

**Structure of Bacterial Cells.**—When examined living in a hanging drop (see p. 72) under the microscope, bacteria appear usually as colorless refractive bodies with or without spores or other more highly refractive areas. It is only by the use of stains that we are able to see more of their structure.

**Capsule.**—Special staining methods (see p. 80) show that many bacteria (some investigators say all) under certain conditions possess a *capsule* (Plate III, Figs. 14 to 16), a gelatinous envelope which is supposed to be formed from the outer layer of the cell membrane. Some bacteria easily develop a much thicker capsule than others. Such forms are known as capsule bacteria. These generally produce a slimy growth on cultivation (*e. g.*, B. mucosus).

Capsules develop best in animal tissues. In cultures, with a few exceptions, they require for their development special albuminous culture media, such as milk, blood serum, bronchial mucus, etc. In ordinary nutrient media or on potatoes the capsule may be visible in the first culture generations when grown from the body, but usually it shows very indistinctly, if at all. The capsule is distinguished by a diminished power of staining with ordinary aniline dyes; therefore, unless special staining methods are used, the bacteria may appear to be lying in a clear unstained area. With certain dyes the inner portion of the capsule stains, giving the bacteria an apparent greater diameter. The demonstration of the capsule is often of help in differentiating between different but closely related bacteria; *e. g.*, some forms of streptococcus and pneumococcus.

**Cell Membrane.**—That all bacteria possess a cell membrane is shown (1) by special staining methods (*e. g.*, flagella stains, see p. 82) and (2) by plasmolysis, demonstrated by placing the bacteria in a 1 per cent. solution of sodium chloride when the central portion (entoplasm?) contracts and separates in places from the membrane (see also p. 58, under Influence of Pressure). In some bacteria the membrane is slightly developed, while in others (*e. g.*, B. tuberculosis) it is well developed. It is different in composition from the membrane of higher plants in not possessing cellulose. In some forms, however, a similar carbohydrate, hemicellulose, has been demonstrated. In certain forms a substance related to chitin, found in the cyst walls of protozoa, has been found. Some observers consider the cell membrane merely a concentrated part of the cytoplasm, similar to the ectoplasm of higher cells. That it is closely related to the living part of the cell is shown by the connection of the organs of locomotion (flagella) with it (Plate III, Figs. 17 and 21).

**The Cell Substance.**—The chief views in regard to the nature and the structure of the cell substance contained within the membrane may be summarized as follows:

1. Bacteria have a definite morphological, more or less centrally situated nucleus (Feinberg, Nakanischi, Shottelius, Swellengrebel and others).
2. Bacteria have no nucleus or differentiated nuclear material (Fischer, Migula, Massart and others).
3. The whole organism, except the membrane which is a delicate layer of cytoplasm, is a nucleus (Bütschli, Löwit, Boni and others).
4. The nuclear material is in the form of distributed chromatin granules throughout the cytoplasm (Hertwig, Schaudinn, Guilliermond, Zettnow and others).
5. A variety of the fourth view is that bacteria possess both chief elements of a cell, namely, cytoplasm and karyoplasm, but that these are so finely mixed that they cannot be morphologically differentiated (Weigert, Mitrophanow, Gotschlich).
6. Another view advanced, which is a variation of the views 3, 4 and 5, is that the bacterial cell is a relatively simple body—a cytode in Haeckel's sense, or the plasson of Van Beneden—which possesses both chromatin and

plastin, the relative amounts of these chief substances of a cell corresponding more to the amounts found in the nuclei of higher cells than in their cytoplasm (Ruzicka,[1] Ambrôz[2]).

The last two authors call attention to the fact that both nucleus and cytoplasm in the higher cells are composed of a mixture of chromatin and plastin and that the chief difference between the two mixtures is one of amount and not of kind.

Our own studies of the structure of bacteria lead us to agree with the views expressed in Nos. 4 and 6 of the above summary—that is, bacteria possess both chief elements of a cell, namely, chromatin and plastin, and according to the stage of growth and division (varying with species) the chromatin may be in the form of morphological granules or may be so finely divided and mixed with the plastin as to be indistinguishable from it. (See Plate III, Figs. 1, 2, 5, 6, 7.)

**Metachromatic Granules** (Plate III, Figs. 1 to 13).—These granules appear in unstained bacteria as light-refracting, in stained preparations, as deeply stained areas. They have a great affinity for dyes, and so stain readily and give up the stain with some difficulty. With complex stains they show a greater affinity than the rest of the bacillus for certain constituents of the stain—*e. g.*, with polychromic methylene blue they take up more of the azure, thus appearing red as does the stained nuclein in morphological nuclei. In certain bacteria, such as the diphtheria bacilli, they are especially well marked in young, vigorous cultures. Here they have diagnostic value. At least some of these granules are nuclear in character.

Certain other granules which take up stains readily, and others still which absorb stains with difficulty, are of the nature of starch, of fat or of other food products. Meyer[3] has described some as being composed of volutin, a protein characterized by insolubility in alcohol and solubility in water, acids and alkalis. Certain saprophytic forms have sulphur, others iron granules.

**Organs of Motility.**—The outer surface of spherical bacteria is almost always smooth and devoid of appendages; but that of the rods and spirals is frequently provided with fine, hair-like appendages, or *flagella*, which are organs of motility (Plate III, Figs. 18 to 21). These flagella, either singly or in tufts, are sometimes distributed over the entire body of the cell, or they may appear only at one or both ends of the rod. The polar flagella appear on the bacteria shortly before division. The flagella are believed to be formed from the outer cell layer (ectoplasm) or possibly from the capsule, though they have been described by certain authors as arising in endoplasmic granules. So far as we know, the flagella are the only means of locomotion possessed by the bacteria. They are not readily stained, so special staining agents are required for this purpose (see p. 82). The envelope of the bacteria, which usually

[1] Cytologie der sporenbildenden Bakterien, etc., Centralbl. f. Bakt., 1909, II. Abt., vol. xxvii.

[2] Entwickelungszyklus des B. nitri n. sp., etc., Centralbl. f. Bakt., etc., I. Abt., orig., 1909, li, 193 (with bibliography on structure and development of bacteria).

[3] Flora, 1908, p. 95.

remains unstained with the ordinary dyes, then becomes colored and more distinctly visible than is commonly the case. Occasionally, however, some portion of the envelope remains unstained, when the flagella present the appearance of being detached from the body of the bacteria by a narrow zone. In stained cultures of richly flagellated bacteria peculiar plaited masses sometimes are observed, consisting of flagella which have been detached and then matted together. Bacteria may lose their power of producing flagella for a series of generations. Whether this power be permanently lost or not we do not know.

Bacteria are named according to the number and position of the flagella they possess as follows: *Monotricha* (a single flagellum at one pole, *e. g.*, cholera spirillum); *amphitricha* (a flagellum at each pole, *e. g.*, many spirilla); lophotricha (a tuft of flagella at one pole,[1] *e. g.*, Spirillum undula); *peritricha* (flagella projecting from all parts of surface, *e. g.*, B. alvei, B. typhosus, and others) (Plate III, Figs. 18–21).

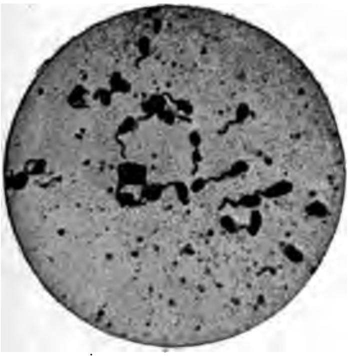


FIG. 2.—Bacilli showing one polar flagellum.

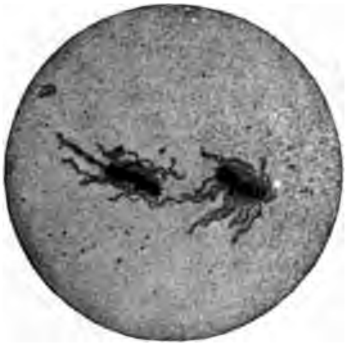


FIG. 3.—Bacilli showing multiple flagella.

So far, in only a few bacteria (*e. g.*, the largest spirilla) have flagella been demonstrated during life, and then only under special conditions (see K. Reichert for bibliography). We have, however, an organism belonging to the B. alvei group, which shows its flagella very distinctly during life when a small portion of the viscid growth in a liquefying Löffler blood-serum tube is transferred to a hanging mass of agar (p. 72) and examined under high magnification. The flagella on this organism may also be seen with dark-field illumination. Reichert claims that all motile bacteria show their flagella by this method.

**Spores.**—These important structures of the bacterial cell are described in detail under Physiological Characteristics of Bacteria.

**Physiological Characteristics of Bacteria.**—The essential physiological activities of bacteria are: motility, growth, reproduction, and spore formation.

**Motility.**—Many bacteria when examined under the microscope are seen to exhibit active movements in fluids. The movements are of a varying character, being described as rotary, undulatory, sinuous, etc.

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[1] Some investigators consider that every flagellum is essentially a tuft, composed of many small fibrils.

Definite progressive movement may be very rapid, or it may be so slow that it may be difficult to say positively whether there is any actual motility or whether the organism shows only molecular movements—so-called "*Brownian*" movements, or *pedesis*—a dancing, trembling motion possessed by all finely divided organic particles. In order to decide definitely with regard to the motility of any bacterial preparation, it is well to make two hanging drops. To one, 5 per cent. of formalin is added, which, of course, kills the organism. If, now, the live culture shows motility, which is not shown by the killed culture, it is an indication that one is dealing with a motile culture. Very young cultures, of but three or four hours' development, in neutral nutrient bouillon should be examined at a temperature suitable for their best growth. Not all species of bacteria which have flagella exhibit at all times spontaneous movements; such movements may be absent in certain culture media and at too low or too high temperatures, or with an insufficient or excessive supply of oxygen; hence one should examine cultures under various conditions before deciding as to the non-motility of any organism.

The highest speed of which an organism is capable has been approximately estimated with some forms, and the actual figures show an actual slow rate of movement, though, comparatively, when the size of the organism is considered, the movement is rapid. Thus, the cholera spirillum may travel for a short time at the rate of 18 cm. per hour.

Movement is influenced by many factors, such as chemicals (the oxygen in the air especially), heat, light, and electricity. The tactile property which enables microörganisms to take cognizance of various forces is known as *taxis;* when forces attract, the phenomenon is known as positive taxis and when they repel, it is called negative taxis. Chemotaxis, or the effect of chemicals, is taken up in detail on page 60.

**Growth and Reproduction.**—Under favorable conditions bacteria grow rapidly to a certain size, more or less constant for each species, and then divide by fission into approximately equal halves. The average time required for this cycle is twenty to thirty minutes. Probably in all species the nuclear material divides first. This is certainly the case in the group to which the B. diphtheriæ belongs, where division of the nuclear granules may be observed in the living organism before the characteristic snapping of the cell body and where division into equal halves seldom occurs.

According to our observations on the living cell of members of this group, division takes place at a point occupied by a metachromatic granule (Fig. 4). Before division of the cell body the metachromatic granule, which appears to contain nuclear substance, elongates and shows a darker line at or near its center. This seems to divide and form two lines, each of which has at a point near the surface a very tiny, refractive granule, staining deeply with chromatin stains. Between these two lines the cell body suddenly divides with a snap, like the opening of a jack-knife, division beginning at the point between the two tiny granules, and the two new cells remain for a variable time attached at opposite points, thus giving the V-shaped forms. Kurth and Hill also called attention to division by snapping in members of the diphtheria-bacillus group, though neither recognized the relation between the position of the metachromatic granules and the point of division. The tiny granules are probably similar to the cell-partition granules described by various observers.

It is not often that the favorable conditions mentioned above for the production of equal and rapid division obtain for any time, since even in pure cultures bacteria in their growth soon produce an environment unfavorable for further multiplication. Several factors help to make this environment: (1) The using up of suitable food and moisture; (2) the disintegration of food substances into various injurious products, such as acids and alkalis; (3) in mixed cultures the overgrowth of one or more varieties. As these unfavorable conditions are more or less constantly present, we seldom see such absolute symmetry in the growth and division of bacteria as is usually described. In fact, even under ideally favorable conditions (*e. g.*, rapid successive transfers from young cultures on the most favorable food medium), we would never see absolutely equal fission among bacteria; and in some species, notably the diphtheria group, division is extremely irregular even in our usual twenty-four cultures on favorable media.

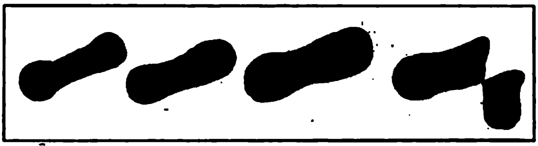

FIG. 4.—Successive stages in division of Bacillus diphtheriæ, showing relation of line of division to metachromatic granule. Continuous observation of living bacillus drawn without camera lucida. (Williams.)

*Involution and Degeneration Forms.*—It follows, from the conditions considered above, that, as cultures grow older or when media unfavorable to equal division are used, the bacteria may show extremely irregular forms, absolutely different from the young forms, such as long threads or filaments with irregular thickenings, coccus forms from bacilli and spirilla which have divided without increasing in length, bacillar forms from cocci which have grown without dividing, and apparently branched forms from many varieties of bacilli and spirilla. These have been called *involution* or *degeneration forms.*

In our study of the so-called branched forms of the diphtheria bacillus we have observed the following interesting fact: Under certain conditions, marked apparent branching appears at a definite time in the age of the culture. The conditions are: slightly disturbed growth in pellicle on nutrient broth. When such pellicles are examined every day they are found to contain, on the sixth to the twelfth day, varying chiefly with the amount of disturbance, many large intensely staining forms with one to several apparent branches and many large metachromatic granules (Figs. 5 and 6). The facts that these forms were the only ones to show active growth and division when examined on a hanging mass of agar and that in such growth the metachromatic granules seem to fuse (Fig. 6) before fission led us to suppose that these forms represent a primitive sexual process, a sort of autogamy. Schaudinn[1] (Fig. 7) has reported a primitive conjugation (autogamy) and a relationship between the chromatin granules, or nuclear substance, and the spores in certain bacteria.

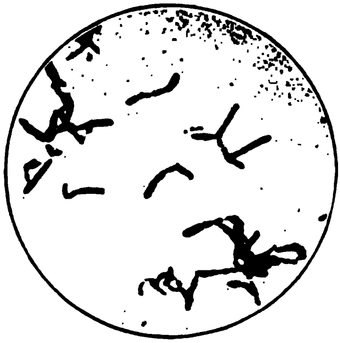

FIG. 5.—Bacillus diphtheriæ "No. 8" from nine days' broth pellicle, showing many "branched" forms. Stained with carbolfuchsin. × 1500 diameters.

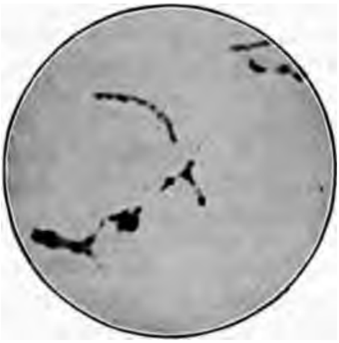

FIG. 6.—Bacillus diphtheriæ "No. 8" from ten days' broth pellicle, showing longitudinal fusion and position of metachromatic granules. Stained with Löffler's methylene blue. × 2000 diameters.

Irregularities in division during the regular life cycle of the typhoid-dysentery group have been reported by Hort.

*Incomplete Segmentation.*—Although elongation in the greater diameter and complete division at right angles to this is the rule for the majority of bacteria, there are certain groups, which, instead of becoming separated from each other as single cells, tend to produce an incomplete segmentation, the cells remaining together in masses, as the sarcinæ,

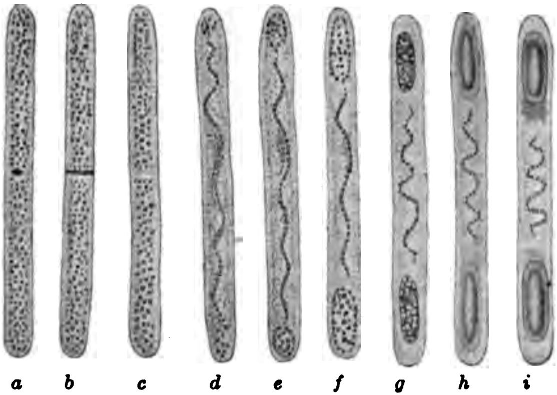

FIG. 7.—Bacillus bütschlii: *a* to *c*, incomplete division of the cell; *d* to *f*, gradual collection of chromatin granules at ends of cells; *g* to *i*, formation of end spores from these chromatin end masses. (After Schaudinn.)

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[1] Beiträge zur Kenntnis der Bakterien, etc., Arch. f. Protistenk, 1902, i, 306, and 1903, ii, 416.

for example, which divide more or less regularly in three directions. The indentations upon these masses or cubes, which indicate the point of incomplete fission, give to these bundles of cells the appearance of a bale of materials. The rod-shaped bacteria have never been seen to divide longitudinally.

*Spore formation* must be distinguished from vegetative reproduction. This is the process by which the organisms are enabled to enter a stage in which they resist deleterious influences to a much higher degree than is possible for them to do in the growing or vegetative condition. It is true that in all non-spore-bearing cultures a certain proportion of the bacteria are more resistant than the average. No marked difference in protoplasm, however, has been noted in them other than the ability to stain more intensely and sometimes to show strong metachromatic areas. The difference between these and the less resistant forms is not great. Some have believed that this resistance is due to certain bodies called *arthrospores*, which are abnormally large cells with, usually, a thickened cell wall and increased staining properties, formed as a rule in old cultures. Foulerton and others have described similar forms in some of the higher bacteria and consider them spores. (See under Nocardia.)

The *true spores* (endospores) of the lower bacteria are definite bodies. These are strongly refractile and glistening in appearance, oval or round in shape, and composed of concentrated protoplasm developed within the cell and surrounded by a very dense envelope (Plate III, Figs. 22 to 25). They are characterized by their power of resisting the injurious influences of heat, desiccation, and chemical disinfectants up to a certain limit (see chapter on Disinfection). Spores also stain with great difficulty. (See page 81 for details.)

The production of endospores in the different species of bacteria, though not identical in every instance, is very similar. The conditions under which they are produced in nature are supposed to be similar to those observed in artificial cultures, but they may not always be similar, hence we must not consider as absolutely settled that a bacterium is a non-sporebearer because in the laboratory it has not been seen to form spores. Usually when growth does take place the formation of spores in any species is best observed in a streak culture on nutrient agar or potato, which should be kept at the temperature nearest the optimum for the growth of the organism to be examined. At the end of twelve, eighteen, twenty-four, thirty, thirty-six hours, etc., specimens of the culture are observed, first unstained in a hanging drop or on an agar mass, and then, if round or oval, highly refractile bodies are seen, stained for spores. Each bacillus, as a rule, produces but one spore, and more than two have never been observed.

Motile bacteria usually come to a state of rest or immobility previous to spore formation. Several species first become elongated. The anthrax bacillus does this, and a description of the method of its production of spores may serve as an illustration of the process in other bacteria (Plate III, Fig. 22, *A–E*). In the beginning, the protoplasm of the elongated filaments is homogeneous, but after a time it becomes turbid and finely granular. These fine granules are then replaced by a smaller number of coarser granules, the so-called sporogenous granules supposed to be chiefly nuclear in nature, which by coalescence finally amalgamate into a spherical or oval refractive body. This is the spore. As soon as the process is completed there may appear between each two spores a delicate partition wall. For a time the spores are retained in a linear position by the cell membranes of the bacilli, but these are later dissolved or broken up and the spores are set free. Not all the cells that make the effort to form spores, as shown by the spherical bodies contained in them, bring these to maturity; indeed, many varieties, under certain cultural conditions, seem to lose altogether their property of forming spores.

The following are the most important spore types: (*a*) the spore lying in the center of the cell; which may be much distended in its central portion, giving it a spindle shape or clostridium, *e. g.*, Bacillus butyricus; (*b*) the spore lying at the extremity of a cell much enlarged at that end—the so-called "head spore" or plectridium, *e. g.*, the tetanus bacillus; (*c*) the spore lying eccentrically, *e. g.*, B. œdematis (Plate III, Fig. 25, *A-C*).

According to Schaudinn[1] and others, in certain spore-bearing bacteria the spore formation is part of a sexual-like process. (See under Reproduction.)

The *germination of spores* takes place as follows (Plate III, Figs. 22–24): By the absorption of water they become swollen and pale in color, losing their shining, refractive appearance. Later, a little protuberance is seen upon one side (equatorial or central germination) or at one extremity of the spore (polar germination), and this grows out to form a rod which consists of soft-growing protoplasm enveloped in a membrane, which is formed of the endosporium or inner layer of the cellular envelope of the spore. The outer envelope, or exosporium, is either cast off when it may be seen in the vicinity of the newly formed rod; or it may be absorbed, as is often the case after central germination.

The chief spore formers among the pathogenic bacteria are the anaërobes (tetanus, malignant edema, intestinal bacteria). Only one distinctly pathogenic aërobe produces endospores—the anthrax bacillus.

**The Higher Forms of Bacteria** (see end of Section II).—Some forms grow out into true or false branching threads and thus make a group of organisms intermediate between bacteria and the moulds. These have been called higher bacteria or trichobacteria (see table, p. 26). They show increased complexity of structure and function: (1) in forming irregularly segmented filaments composed of elements similar to those found in the lower forms and showing either true or false branching; (2) in developing certain portions of their substance into reproductive bodies from which the new individuals grow (Plate II, Figs. 15 and 16).

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[1] Beiträge zur Kenntnis der Bakterien, etc., Arch. f. Protistenk, 1902, i, 306, and 1903, ii, 416.

The filaments seen sometimes among the lower forms have independent segments, which may easily separate and grow as tiny unicellular forms, while in the higher forms the filaments in their growth show a certain interdependence of their parts. For example, growth often occurs from only one end of the filament while the other becomes attached to some fixed object.

The members of the higher bacteria which are pathogenic for man have as yet been incompletely studied and classified. The following divisions serve as an attempt at differentiation:

1. *Leptothrix* grows in stiff, almost straight threads, in which division processes are seldom or never observed, and no branching has been seen.
2. *Cladothrix* grows in threads which rapidly fragment and produce false branching, that is, the terminal cell remains partly attached, but is pushed to one side by further growth from the parent thread; thus a Y-shaped growth is produced. Bacillary characteristics appear in old cultures.
3. *Actinomyces* grows in threads with true branching. Spores have been observed. It is characterized by the radiating, wreath-like forms which it alone produces in the living body.
4. *Nocardia* (*Streptothrix*) grows in threads which produce abundant true branching; later there is fragmentation and formation of conidia.

*Reproduction among the Higher Bacteria.*—These forms increase in length for a time and then, at the free ends, or at intervals along the filaments, they produce small rounded cells, called conidia or spores, from which new individuals are formed. The terminal spores may be flagellated after their separation from the parent filament.

The flagellated forms may resemble certain flagellata among the protozoa.

## PROTOZOA.

**Definition.**—A protozoön (the lowest form of life classed in the animal kingdom) is a morphologically single-celled organism composed of protoplasm which is differentiated into cytoplasm and nucleus (or nuclear substance) both of which show many variations throughout the more or less complicated life cycle that each individual undergoes.

The protozoa are of higher grade than the bacteria because of their greater complexity in structure and life cycle (Plate IV).

Their shape and size vary so widely that no general description will fit all types. Some forms are small enough to pass through a Berkefeld filter, while the largest varieties described are about ⅔ inch long.

**The Cytoplasm.**—The cytoplasm consists of a mixture of substances, the most important of which belong to the proteins. It is more or less fluid, but, because of differences in the density and solubility of the several parts, it often presents an alveolar, linear, or granular appearance, which may come out clearly in fixed and stained specimens, but is usually not well seen in the living cells.

**Ectoplasm and Entoplasm.**—Frequently the protozoön cytoplasm is differentiated into a concentrated, viscid, more homogeneous, or hyaline outer layer called the *ectoplasm* and a more fluid granular central portion called the *entoplasm*. These two portions have different functions. The ectoplasm helps form the various organs (organelles) of motion, contraction, and prehension such as pseudopods (false feet), flagella (whip-like threads), cilia (hair filaments), suctorial tubules (through which food passes), and myonemes (contractile fibrils found in ciliates, gregarines, and a few flagellates). The entoplasm digests the food and contains the nucleus, as well as various granules and vacuoles. Some vacuoles serve as food digesters, and hence contain digestive ferments. The so-called contractile vacuoles which periodically fill and empty themselves may be considered as excretory organelles.

Other substances are seen from time to time in the entoplasm, such as bacteria, red blood cells, fatty granular pigments, bubbles of gas, crystals, etc.

**The Nucleus.**—The simplest morphological nucleus is a vesicular body which is differentiated from the cytoplasm by its essential constituent, chromatin, so called because it has a strong affinity for certain basic staining materials. Chromatin consists mostly of nuclein and appears in the form of smaller or larger granules, masses, or rods.

Generally, the chromatin particles are mixed with a second less intensely staining substance with more of an affinity for acid stains called plastin or paranuclein, similar to the substance from which the true nucléolus of the metazoön cell seems to be formed. This substance may appear in one or more distinct, rounded bodies. Most of the chromatic substances of the nucleus in many protozoa are often massed together in an intensely staining ball-like body called the *karyosome* which undergoes various cyclic changes during the growth and development of the organism. The centrosome is generally imbedded in the karyosome. The chromatin and plastin lie imbedded in a third substance in the form of an achromatic network called linin, which is closely related to the cytoplasmic network. There may or may not be a definite nuclear membrane. Sometimes there is no definitely structured nucleus, but the nuclear substance in the form of small chromatin masses or granules is distributed throughout the cytoplasm (the so-called "distributed nucleus") similar to that seen in bacteria.

*Somatic and Generative Chromatin.*—Some chromatin substances of the cell have physiological properties different from others. At times substances which have only vegetative properties are active, forming the so-called somatic or trophic chromatin; at other times, substances appear during sexual activities called generative or sexual or idiochromatin, and from these the vegetative (somatic) chromatin for the new cells is again formed. In the ciliata both these chromatin elements are present as distinct morphological bodies during the entire life of the organism, the somatic form in the macronucleus and the generative form in the micronucleus.

*Chromidia.*—The chromatin elements, in the form of granules, small irregular masses, threads, network, etc., which at certain stages pass from the nucleus into the cytoplasm, or which at times are, possibly, formed in the cytoplasm, were named "Chromidien" by R. Hertwig, who in 1899 first described their appearance. Their function in generative processes was demonstrated in 1903 by Schaudinn. During their formation the nucleus may entirely disappear, so that morphologically the cell may be considered non-nuclear. At a definite time thereafter new typical nuclei may be formed from these chromidial substances.

*Locomotor Nucleus* (*Kinetic Nucleus*).—In flagellates still another definite physiological chromatin is seen in the small body called the kinetic nucleus, which is either apart from or merged into a smaller body, the blepharoplast, forming the root of the flagellum. The kinetic nucleus is so called because it produces the locomotor apparatus. Both the kinetic and trophic nuclei may contain somatic and generative chromatin at the same time.

**The Centrosome.**—This is a small body which is always present in metazoan cells, playing an important part in cell division, but it has not been demonstrated as a morphological entity in many varieties of protozoa; part of the karyosome, however, may take its place, or there may always be a true centrosome within the karyosome. Whenever a centrosome appears in protozoa, it has its origin in the nucleus, resembling in this the kinetic nucleus and blepharoplast. All these four bodies (centrosome, blepharoplast, kinetic nucleus and karyosome) therefore may be considered as having a similar morphological origin.

**Physiological Characteristics of Protozoa.**—In common with all other living organisms protozoa possess the characteristics of motility, nutrition, respiration, and reproduction.

**Motility.**—All protozoa react in certain characteristic ways toward chemical, mechanical, and electrical stimuli. Many are affected by light, while probably none reacts to sound. They manifest the reaction usually by motion of some sort. Most animal parasites, especially the higher forms, exert a positive taxis for leukocytes, principally for the large mononuclears and the eosinophiles. This fact is made use of in clinical diagnosis.

**Nutrition.**—Many protozoa, especially the pathogenic forms, absorb fluid food directly through the body wall; but the majority take in solid food, such as small animal or vegetable organisms and organic waste, some through more or less definite regions of the body, others through any part of the surface by extending pseudopodia and entirely surrounding the food object, forming a so-called gastric vacuole.

After the food is digested the waste products are excreted, sometimes by osmosis, generally through special structures as the contractile vacuoles which regularly eject fluid substances to the outside of the organism.

**Respiration.**—It is supposed that the contractile vacuole has a respiratory as well as an excretory function. The interchange of gases is always going on, if not through a contractile vacuole, then by osmosis through any part of the wall.

**Growth and Reproduction.**—Under favorable conditions, new protoplasm is constructed rapidly, and the mass increases faster than the surface which, according to Spencer, initiates cell division. The changes generally appear first in the nucleus. The simplest variety of reproduction is a two-celled fission which may be either longitudinal or transverse, either of which may be direct (amitotic) or indirect (mitotic). A modification of equal fission is the so-called budding which may be single or multiple. When growth occurs so that fission is for a time incomplete, one cytoplasm containing several nuclei which finally separate into as many daughter organisms, the process is called multiplicative reproduction, or brood formation or internal budding. In the most extreme cases of multiplicative reproduction as it occurs among sporozoa, the mother-cell with its nucleus separates simultaneously into large numbers of tiny daughter-cells. Such a process, when it occurs without conjugation and encystment, is called schizogony and the new cells are called merozoites. When such a multiplicative division occurs (generally after fertilization) within a cyst, it is spoken of as sporogeny and the new cells are called sporozoites.

*Sexual Phenomena.*—Sexual phenomena (syngamy) fundamentally similar to those seen in metazoa have been observed in all groups of protozoa studied. The reproduction by the usual division or budding is interrupted at certain times in the life history of each organism and individuals come together in such a way that their nuclei fuse, usually after having undergone characteristic reduction divisions.

When the union is permanent, we speak of it as copulation and liken the process to that of the fecundation of the ovum by a spermatozoön. When the union is transient we call it conjugation. Here the two cells fuse for a time when the nuclei interchange protoplasm and then the cells separate and each one continues to grow and divide independently. When in a partly divided cell or in an apparently single cell, two nuclei, after undergoing reduction division, or its like, fuse, the process is called autogamy.

*The developmental cycle* of a protozoön consists of all the changes which occur in its growth from one act of fertilization to another (Plate IV, Fig. III). Many protozoa carry on the sexual part of their life cycle in one host and the asexual part in another (*e. g.*, malarial organisms).

**Cyst Formation.**—If protozoa do not get the required amount of water or air or suitable food, they cease their special movements, round out into more or less of a sphere and form a resisting membrane of chitin within which they may live for a long time, withstanding periods of desiccation, extreme heat and cold, and they may be blown about as dust until they find conditions again favorable for renewed growth; then water is absorbed, the cyst is ruptured and active life begins anew.

In parasitic forms encystment plays an important part in the passage from the old host to the new. The majority of forms would not be able to exist outside of the body of the host without having some protective membrane. The cyst may be formed simply for protection from drought, etc., when it is called a hypnocyst, from which the organism may emerge in about the same form as when it encysted; or the cyst may precede reproduction by spore formation or simple division, when it is called a sporocyst. In either case it may consist of a simple wall or it may be formed of several walls to enable it to resist prolonged desiccation, when it is called a resting cyst.

**Characteristics of Each of the Four Groups of Protozoa.—Flagellata.**—Flagellata are protozoa which move in the adult forms by one or several flagella or whip-like processes. If pseudopodia develop, they are transitory.

Generally the flagella arise from the anterior part of the organism, and in motion the larger ones (primary flagella) are directed forward, while smaller ones (secondary flagella) are directed backward, acting as rudders. Certain flagellata possess a modification of their bodies in what is called the undulating membrane, which consists of a fluted protoplasmic process attached along one side of the organism, the free edge of which is prolonged as the flagellum. It has been shown that flagella are not simple protoplasmic processes, but that they have more or less of a framework of elastic fibers as well, hence their power in locomotion can be better understood. Except with special stains, which bring out these fibers, they appear homogeneous.

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## EXPLANATION OF PLATE IV.

Partly schematic. Rearranged and drawn by Williams. All stained by Giemsa.

I. Flagellates.

Fig. 1.—Illustrating one flagellum. Leishmania: *A*, intracellular form; *B*, cultural forms.

Fig. 2.—Illustrating undulating membranes: *A*, Trypanosoma lewisi; *B*, Trypanosoma brucei; *C*, Trypanosoma gambiense.

Fig. 3.—Illustrating two flagella. Bode lacertæ (after Prowazek).

Fig. 4.—Illustrating four flagella. Trichomonas.

II. Amebæ.

Illustrating points considered differential in the two chief types of amebæ (entamebæ) described as parasitic in human beings.

Fig. 1.—Entameba coli, vegetative stage.

Fig. 2.—Dividing nucleus.

Fig. 3.—Entameba coli cyst containing eight nuclei.

Fig. 4.—Entameba histolytica, vegetative stage.

Fig. 5.—Four-nucleate cyst.

III. Sporozoa.

*A*, description of Figs. 1 to 16. (After Schaudinn.) The life cycle of Eimeria schubergi. In 1, the sporozoites, becoming free by bursting the sporocysts, pass out through an aperture in the wall of the oöcyst, and are ready to enter the epithelial cells of the host. 2 to 6 represent the asexual reproduction or schizogony, commencing with infection of an epithelial cell by a merozoite or a sporozoite; the merozoite after stage 6 may start again (5) at stage 2, as indicated by the arrows, or it may go on to the formation of gametocytes (9 to 11). 9 to 11 represent the sexual generation, the line of development becoming split into two lines—male (♂) and female (♀)—culminating in the highly differentiated gametes, which conjugate and become again a single line, shown in 12–14. The zygote thus formed goes on to the production of spores, 15 and 16. 2 and 3 represent epithelial cells showing penetration of a merozoite or a sporozoite and its change into a schizont; 4, the nucleus of the schizont divided into numerous daughter-nuclei; 6, segmentation of the schizont into numerous merozoites, about a central mass of residual protoplasm, which in this figure is hidden by the merozoites; 5, merozoites passing to reinfect host cell and repeat the process of schizogony; 7, 8, merozoites to be differentiated into male and female gametocytes; 9, the two gametocytes within a host cell (the microgametocyte (♂) has fine granulations; the macrogametocyte (♀) has coarse granulations; 11, a female gametocyte undergoing maturation; 13, mature macrogamete, free from the host cell, and sending a cone of reception toward an approaching microgamete. In 12 the nuclei of the last stage have become microgametes, each with two flagella. The free microgametes are swimming to find a macrogamete. 14, the zygote (fertilized macrogamete), surrounded by a tough membrane or oöcyst, which allows no more microgametes to enter and containing the female chromatin, which is taking the form of a spindle, and the male chromatin in a compact lump; 15, the nucleus of the zygote divided—the nuclei of the sporoblasts; in 16 the four sporoblasts become distinct, leaving a small quantity of residual protoplasm; each sporoblast has formed a membrane, the sporocyst. Within each sporocyst two sporozoites form about a sporal residuum.

*B*, Babesia infecting red blood cells: 1, pear-shaped bodies; 2, dividing forms; 3, eight pear-shaped bodies in a cell; 4, irregular ring-like bodies; 5, large, irregular body; 6, body with a flagellum-like projection.

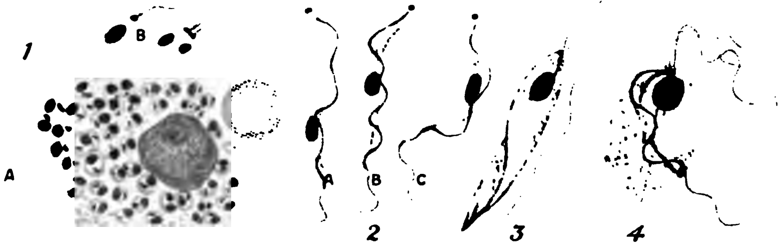
IV. Ciliates.

Fig. 1.—Balantidium coli (after Hartmann): *A*, adult form; *B*, *C*, dividing forms; *D*, conjugating forms.

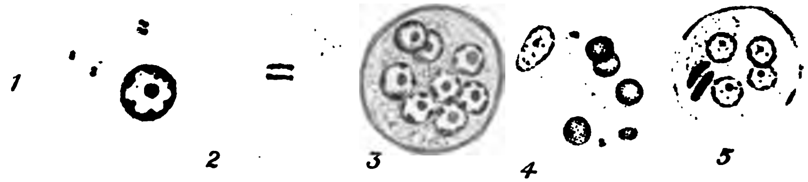
# PLATE IV

## TYPES OF PROTOZOA

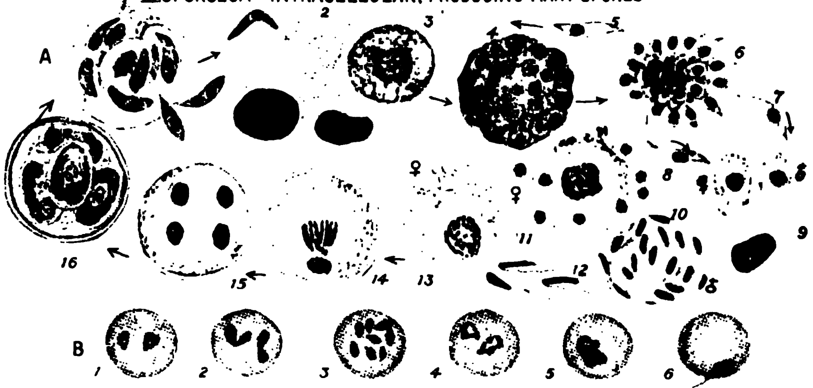
### I. FLAGELLATES—MOVING BY FLAGELLA



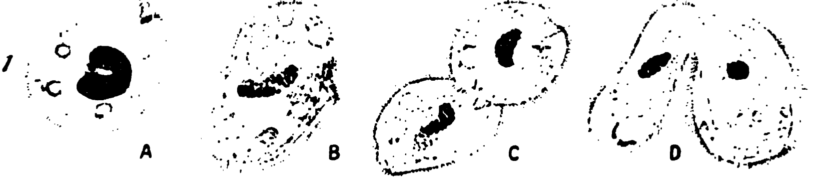
### II. AMEBÆ — MOVING BY PSEUDOPODS



### III. SPOROZOA—INTRACELLULAR, PRODUCING MANY SPORES



### IV. CILIATES—MOVING BY CILIA



A. W. WILLIAMS, DEL.

The flagella arise from some definite place in the cytoplasm, sometimes from a distinctly differentiated chromatic body which has been given various names, such as blepharoplast, kinetic nucleus or centrosome, sometimes near this from a basal granule, microsome, diplosome, or flagellum root, sometimes directly from the nucleus. The basal granules seem to be derived primarily from the kinetonucleus, and may be considered from a physiological standpoint as a part of the motor nuclei.

The body of the flagellates is generally more or less elongated and, except in most primitive ones, is fixed in its outline. The latter characteristic is chiefly due to the fact that the organisms usually possess definite though delicate membranes containing elastic fibrils. The cytoplasm is usually not differentiated into an ento- and ectoplasm. It often contains one to several contractile vacuoles, as well as food vacuoles, and there is frequently a definite opening or cytostome for the entrance of food. There are usually many granules and inclusions of various kinds scattered throughout the cytoplasm, and myoneme striations are seen in some forms. The nucleus, as a rule, situated anteriorly, varies much according to different species and to different stages of development.

The flagellata multiply either in the free motile condition or after encystment. In the first case, as a general thing, they divide longitudinally. The basal granules divide with the nuclei and the flagella of the daughter organisms are usually formed anew. Multiple division is also observed. In the second case the flagellata may or may not conjugate before they encyst. Then they divide within the cyst.

The sexual cycle varies much in different species. Isogamy has been noticed between fully grown individuals as well as between smaller forms. The union of different-sized forms, or anisogamy, has also been observed. Also autogamy is said not to be infrequent. It is claimed that certain of the flagellates pathogenic for man require a second host for the development of their sexual cycle.

The flagellates are subdivided into several orders, in only two of which are forms found which are pathogenic for man. (See Part II.) The different types of pathogenic flagellates are shown in Plate IV, I, Figs. 1 to 4. Among the most important pathogenic forms are the trypanosomes.

**Trypanosoma.**—The name trypanosoma (boring animal) was given by Gruby, in 1843, to certain free-swimming hemoflagellates found by him in the blood of frogs. Much later similar flagellates were found in the blood plasma of many different species of vertebrates and in the intestinal tract of several blood-sucking invertebrates. Some of the forms, including those found in man, are pathogenic. A number of the blood-sucking insects are carriers of the diseased species to healthy animals.

Typical trypanosomes are characterized by a comparatively long, spirally twisted body, along one side of which is attached an undulating membrane having a cord-like edge that is continued forward as a free whip (flagellum). The flagellum arises near the posterior end of the organism in a small granule called the basal granule, which may be connected with the blepharoplast, a larger chromatin mass, called also the kinetonucleus because of its control over the motor apparatus. The nuclear apparatus consists of a macro- or trophonucleus, and of the above-mentioned kinetonucleus or blepharoplast, which last functions as a centrosome. The trophonucleus is usually situated near the middle of the organism; it is granular, thick, and egg-shaped, but varies somewhat in size and shape. The cytoplasm is faintly alveolar or granular, varying with age, environment, and possibly species. Toward the straight border of the cell the cytoplasm is more or less striated and in a few species definite myonemes are seen.

Reproduction occurs usually by longitudinal, occasionally by multiple division. The life cycle is not well known. Though transmission occurs through the bites of various invertebrates, notably flies, the few sexual changes described as taking place in the intestines of some of these intermediate hosts have not been fully corroborated. That an intermediate host is not necessary for the continued life of at least one species of trypanosome seems to be proved by the fact of direct transmission of T. equiperdum from horse to horse through coitus.

**Leishmania.**—Another important pathogenic flagellate is Leishmania. In humans this grows chiefly within large mononuclear cells. It shows its flagellated forms in cultures. (Plate IV, Fig. 1. See also Part II.)

**Amebæ.**—Under amebæ (syn., amebida) we include forms composed of naked, simply constructed protoplasm having the power of producing lobose pseudopodia which are used as organs of motion and of nutrition.

The pseudopodia are protoplasmic processes which are projected in irregular succession from different parts of the surface of the cell, producing in this way an irregular motion. The form of the pseudopodia varies considerably in the different species. For instance, there are broad, blunt processes or narrow, less blunted ones, and each may be short or long, single or slightly branched. The cytoplasm may or may not take a share in their formation. The forms, of course, vary within limits according to the condition of the medium in which the amebæ are living. Movements are always called forth by some physical or chemical excitant. When such an excitant is desirable for food the pseudopods flow around it, and it is subsequently absorbed in the cytoplasm of the organism.

The members of this group may possess one nucleus or several. *Ameba binucleata* has two nuclei in the young adult stage, and *Pelomyxa palustris*, living in the bottom ooze of ponds, has an enormous number of nuclei. A marked feature of the nuclear apparatus is the formation of chromidia which, as has already been noted, may play such an important part in sexual reproduction. Generally each ameba at some stage of development has one contractile vacuole, but occasionally some are seen with several or with none.

Saprophytic forms belonging to this order are common. They may be found wherever there are moisture and decaying vegetable matter. The pathogenic forms are not so frequent. Because of the possibility that the still unknown causes of certain diseases (see Rabies and Smallpox) are organisms related to this order, it is especially important to study both saprophytic and pathogenic varieties, since a knowledge of the former which are more easily studied may help us understand obscure points in the life history of the latter.

Notwithstanding the common occurrence of saprophytic forms, the full life history of few of them has been worked out, and until the full cycle of development of any so-called ameba is known it is impossible to say whether that particular form belongs among rhizopoda or whether it is a developmental form of another group, as ameboid forms may occur at some time in the life history of all groups. It is quite possible that some of the organisms described as belonging to this order are really members of entirely different orders. For instance, it is known that the flagellate *Trichomonas* loses its flagella before copulation and crawls about by means of short blunt pseudopods as a typical ameba.

Amebæ reproduce by simple fission, by budding, and by brood formation. In the last case the reproduction is usually preceded by encystment. Two forms have been described most frequently as parasitic in man. The chief differences claimed to exist between them are shown in Plate IV, II, Figs. 1 to 5.

**The Sporozoa.**—The sporozoa are a group of exclusively parasitic protozoa of very widespread occurrence, living in the cells, tissues, and cavities of animals of every class. Generally they are harmless, but some varieties may produce pathological changes and even fatal diseases severely epidemic.

As their name indicates, they are all characterized by reproduction through spore formation, but they exhibit the utmost diversity of structural and developmental characteristics. As a rule, each species is parasitic on one kind of tissue of a particular species of host. They are generally taken into the system in the spore stage either (1) with the food of the host, (2) by the bites of insects, or (3) by inhalation. The spore membranes are dissolved by the fluids of the host, and thus one or more germs or sporozoites are set free to bore into the special cells of the host. Here they grow, some remaining permanently intracellular, others only in the young stages. The latter either pass different phases of their more or less complicated life history in different parts of the body of one and the same host or they pass some phases of their life cycle in the cells of an intermediate host.

The sporozoa vary widely in size as well as in other characteristics. From the smallest, several of which can be contained in a single blood cell, there are all gradations in size up to those that may be seen by the naked eye (*Porospora gigantea*, 16 mm.).

Besides being characterized by the power to produce very many resisting spores, the sporozoa are also characterized by the fact that as a class they possess none of the special organs found in other protozoa for ingesting or digesting solids. Many develop flagella during sexual phases or show ameboid movement during certain stages of their life cycle, but the flagella and pseudopodia are organs of locomotion and not of nutrition. Food vacuoles or contractile vacuoles have not been found. The life cycle of a typical sporozoön is rearranged and condensed from Schaudinn in Plate IV, III, Figs. 1 to 16.

**Ciliata.**—The ciliata (Plate IV) belong to the most complex of the protozoa. They possess a definite entoplasm containing nuclei and food vacuoles, and a definite ectoplasm containing basal granules from which arise the cilia which give the group its name. They have organoid structures which receive the food, some having definite mouth openings and definite places for excreting waste products. The food vacuoles, may contain acid or alkaline digestive products. The nuclear material is differentiated into two forms, a large macronucleus and a much smaller micronucleus. The function of the macronucleus is supposed to be vegetative, and that of the micronucleus reproductive. The macronucleus varies in size and shape and is completely filled with an alveolar chromatin. The micronucleus also varies in size and shape, and except in reproductive phases is generally vesicular in structure, with the chromatin heaped in one mass. Division of the nuclei takes place by mitosis in the case of micronuclei, and by amitosis, as a rule, in the case of the macronuclei. Under conditions unfavorable for growth the ciliata may encyst.

Conjugation seems to be necessary to the life activity of these organisms. The phenomenon of conjugation in the ciliata has been well worked out. The micronuclei play the most important part, whereas the macronuclei simply break up and disappear in the protoplasm.

According to the arrangement of the cilia, the ciliata are divided into the four orders given in the general classification (see p. 26). Among these, the second, the order of the Heterotricha, interests us. In the Heterotricha the cilia are uniform over most of the body, while a specialized set fused into a series of firm vibratory plates is found about the mouth. Only one genus, *Balantidium*, has been observed in man (Plate IV; also Part II).

## CHEMICAL COMPOSITION OF MICROÖRGANISMS.

Quantitatively considered, the bodies of microörganisms consist largely of water, salts (chiefly phosphorus, potassium, chlorine, calcium, iron and sulphur), fats and albuminous substances. There are also present, in smaller quantities, extractive substances soluble in alcohol and in ether. Special varieties contain unusual substances, as wax and hemicellulose in tubercle bacilli. Each variety, furthermore, yields protein substances peculiar to itself, as shown in the effects produced by animal inoculation. At present we know but little concerning the differentiation of these specific substances. This subject will be taken up in detail under Toxins, etc. According to Cramer,[1] many bacteria contain amyloid substances which give a blue reaction with iodine. True cellulose has not been found in bacteria by Vaughan[1] or other workers, but large quantities of a gelatinous carbohydrate similar to hemicellulose have been obtained. Nuclein is found in all microörganisms. The nuclein bases—zanthin, guanin and adenin—have been obtained in considerable amounts. Vaughan found no sodium chloride in his alcoholic extracts. There is a group of bacteria which contains large amounts of sulphur—viz., the *Beggiatoa*—and another group, the *Cladothrix*, is capable of separating ferric oxide from water containing iron.

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[1] Arch. of Hygiene, vols. xii to xxviii.

Microörganisms possess the capacity to a high degree of accommodating their chemical composition to the variety of soil in which they are growing. Thus, *B. prodigiosus*, when grown on potato, contains 21.5 per cent. of dry residue and 2.7 per cent. of ash; when cultivated on turnips it contains 12.6 per cent. of dry residue and 1.3 per cent. of ash. Besides the concentration of the culture, its temperature and age also influence the amount of residue and ash produced. Qualitatively, a variation is shown by the addition of peptone in the culture media which tends to increase the percentage of nitrogenous matter in the microbe, or by the addition of glucose which decreases it.

The chemical composition of the bodies of animal parasites is an almost unexplored field. The ectoplasm and the cyst sacs in general are made up principally of a substance called chitin. Glycogen has been isolated from many forms. Proteolytic enzymes and acid secretion in digestive vacuoles have been demonstrated.

**Microchemical Reactions.**—To a certain degree the chemical composition of the *individual* organism may be studied both in the living and in the dead individual by the addition of the testing substances to a hanging drop or to a spread of such organism and the examination of it under the microscope. Thus, fats have been demonstrated by staining with osmic acid, Sudan III, or Scharlach R., as well as by alcohol-ether extraction.

Of special importance in this regard is the resistance which bacteria possess to diluted alkalies. Inasmuch as the majority of animal tissues are dissolved when treated with alkalies, this method has been adopted for rendering visible unstained bacteria in tissues. (See also Principles of Staining, p. 76.)

## EFFECTS OF SURROUNDING FORCES UPON MICROÖRGANISMS.

**Food.**—Naturally, the effect of food upon organisms is marked. Though the majority of pathogenic microörganisms grow easily on certain artificial foods (culture media), some of them, like most of the protozoa, we have not yet been able to cultivate outside of the body of their host. Those microörganisms which seem to depend entirely upon a living host for their existence are known as *pure parasites;*

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[1] Herter Lectures, 1915.

those which live only upon dead organic (a few on inorganic) substances are called *pure saprophytes;* those which can lead a saprophytic existence, but which usually thrive only within living tissues, are called *facultative saprophytes,* while those that grow usually on dead material, but may grow within living tissues, are called *facultative parasites.* The *pure saprophytes,* which represent the large majority of all microörganisms are not only harmless to living organisms, but perform many exceedingly important functions in nature, such as the destruction of dead organic matter and its preparation for plant food through decomposition, putrefaction and fermentation, while one group (see below, the Nitrifying Bacteria) are constructive in their activities. The *parasites,* on the contrary, may be harmful invaders (pathogenic microörganisms) of the body tissues, exciting by their growth and products many forms of disease. (See chapter on Relation of Microorganisms to Disease.) The substances essential for the majority of those forms which can be grown artificially are organic material as a source of carbon and nitrogen, an abundance of water, and certain salts. Either calcium or magnesium and sodium or potassium salts are usually required, also sulphur and phosphorus salts. Iron is demanded by a few varieties. The demands of microörganisms for food of a definite composition vary considerably. (See chapter on Cultivation of Microörganisms.) The question as to the form of nitrogenous compounds that is best for growth has been studied by Bainbridge,[1] Rettger and his co-workers,[2] and several others. They state that nature proteins are not directly utilized by bacteria. Rettger and coworkers (1916) further state that coagulated albumin shows the same resistance to the direct action of bacteria as do the unchanged native proteins and that proteose and peptone are not attacked, or at least very slowly by the gelatin non-liquefying species, while the organisms elaborating a proteolytic enzyme break up these substances quite readily. They studied later[3] the amino-acids and related simple nitrogenous compounds, and found that while many saprophytes were able to use these substances as a source of nitrogen, few of the pathogens studied showed growth.

While it is true that very wide differences in relative composition and total concentration of food media may have slight effect upon the general development of a given organism, slight changes in composition and reaction of the media often have a great effect upon morphology, rate of growth, motility, and specific products of growth.

**Reaction of Media.**—The reaction of the media as to acidity or alkalinity is of very great importance. Most bacteria, particularly the pathogenic forms grow best on those media that approach neutrality. (See under Reaction in Chapter on Media.) Yeasts and moulds grow best on a slightly acid medium. An amount of acid or alkali insufficient to prevent the development of bacteria may still suffice to rob them of some of their most important functions, such as the production of enzymes. The different effect upon closely allied varieties of bacteria of a slight excess of acid or alkali is sometimes made use of in separating those which may be closely allied in many other respects.

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[1] Jour. Hyg., 1911, xi, 341.

[2] Rettger, Berman and Sturges: Jour. Bact., 1916, i, 15.

[3] Koser and Rettger: Jour. Inf. Dis., 1919, iv, 301.

**Action of One Species Upon Another.**— In nature, microörganisms usually occur in mixed cultures (*e. g.*, in water, milk, intestinal contents of all animals), and here we may see antagonistic action in the prevalence of one species over others (*e. g.*, the lactic acid formers in the intestines), or coöperative action in the equal and luxuriant growth of two or more species (*e. g.*, pneumococcus and influenza bacillus in the lungs).

Experimentally, the existence of antagonisms can be demonstrated by inoculating alternate streak cultures of various bacteria on gelatin or agar media. It is found that many species will grow not at all or only sparingly when in close proximity to some other species. This antagonism, however, is often only one-sided in character and may be due to such definite causes as the production of acid from the fermentation of sugars. Again, when gelatin or agar plates are planted with a mixture of two species of bacteria, it may be observed that only one of the two grows. A third method of making this experiment is simultaneously to inoculate the same liquid medium with two species, and then to examine them later, both microscopically and by making plate cultures; not infrequently one species may take precedence over the other which after a time it may entirely overcome.

The symbiotic or coöperative action of microörganisms may be demonstrated experimentally in the following examples:

(*a*) Pneumococci, when grown together with a bacillus obtained from the throat, produce very large, succulent colonies. The influenza bacillus, which will not grow alone upon ordinary nutrient agar, will grow well there in the presence of certain other bacteria. Some anaërobic species grow even with the admission of air if only some aërobic species are present (tetanus bacilli with diphtheria bacilli).

(*b*) Certain chemical effects, as, for instance, the decomposition of nitrates, cannot be produced by many species of bacteria alone, but only when two are associated.

**Behavior toward Free Oxygen and Other Gases.**—The majority of microörganisms absolutely require free oxygen for their growth, but a considerable minority fail to grow unless it is excluded. This latter fact, noted first by Pasteur, led him to divide germs into aërobic and anaërobic forms. Between these two groups we have those that can grow both with and without free oxygen. Organisms that can grow under conditions other than the most favorable are called facultative organisms.

(*a*) **Aërobic Organisms.** — Growth is most abundant only in the presence of free oxygen. The slightest restriction of air lessens development. The anthrax bacillus, the hay bacillus and many common air bacilli are examples of this class. Spore formation, especially, requires the free admission of air.

(*b*) **Anaërobic Organisms.**—Growth and spore formation only on the practical exclusion of free oxygen. Among this class of organisms are the bacillus of malignant edema, the tetanus bacillus, the bacillus of symptomatic anthrax, and many soil bacteria. Exposed to the action of oxygen, the vegetative forms of these bacteria are readily destroyed; the spores, on the contrary, are very resistant. Anaërobic germs being deprived of free oxygen—the chief source of energy used by the aërobic species to oxidize the nutritive substance in the culture media—are dependent for their oxygen upon decomposable substances, such as grape-sugar.

(*c*) **Facultative Anaërobic and Facultative Aërobic Organisms.**—The greater number of aërobic germs, including most of the pathogenic species, are capable of withstanding, without being seriously affected, some restriction in the amount of free oxygen admitted (facultative anaërobes), and some grow equally luxuriantly under both conditions. Life in the animal body, for example, as in the intestines, necessitates existence with diminished supply of oxygen. If in any given variety of bacteria the amount of oxygen present is unfavorable, there will be more or less restriction in some of the life processes of this variety, such as pigment and toxin production, spore formation, etc. Pigment formation almost always ceases with the exclusion of free oxygen and proteolysis of facultative anaërobes is lessened.

It has been observed not infrequently that certain species which on their isolation at first show more or less anaërobic development—that is, a preference to grow in the depth of a shake agar-tube culture, for instance—after a while seem to become markedly aërobic, growing abundantly on the surface of the medium (facultative aërobes).

Those organisms that grow best or grow exclusively when the oxygen is only partly removed are called *micro-aërophilic organisms.*[1]

**Other Gases.**—While all facultative organisms as well as strict anaërobes grow well in nitrogen and hydrogen, they behave very differently toward carbonic acid gas. A large number of these species do not grow at all, being completely inhibited in their development until oxygen is again admitted—for example, B. anthracis and B. subtilis and other allied species. It has been found in some species, as glanders and cholera, that the majority of the organisms are quickly killed by $CO_2$, while a few, such as staphylococci, offer a great resistance, rendering impossible complete sterilization by means of this gas. The streptococcus as well as the staphylococcus exhibits a scanty growth. A mixture of one-fourth air to three-fourths carbonic acid gas seems to have no injurious effect on bacteria which cannot grow in an atmosphere of pure $CO_2$. Under pressure $CO_2$ is more effective (page 58).

Sulphuretted hydrogen in large quantity is a strong bacterial poison. Even in small amounts it kills some bacteria.

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[1] Lyon, in Science, 1917, lxv, 19, suggests that the word oligaërobic is better than the word micro-aërophilic.

**Effect of Temperature.**—Some form of microbic life is possible within the limits of 0° and 70° C. The maximum and minimum temperature for each individual species ordinarily lies from 10° to 30° C. apart, and the optimum covers about 5° C. Usually the temperature of the soil in which the germs are deposited is the controlling factor in deciding whether growth will or will not take place. Thus, nearly all parasitic microörganisms require for development a temperature near that of the body of their host, while many saprophytic forms grow best at temperatures lower than 37° C. Microbes when exposed to lower temperature than suffices for their growth, while having their activities decreased, may not be otherwise injured unless actually frozen for a certain time; when exposed to higher temperatures than allows of growth the life of the organism is more or less quickly destroyed. Sudden marked changes in temperature are detrimental.

Microörganisms have been classified according to the temperatures at which they develop, as follows:

*Psychrophilic Microbes.*—Minimum at 0° C., optimum at 15° to 20° C., maximum at about 30° C. To this class belong many of the water microörganisms, such as the phosphorescent bacteria in sea-water; and many moulds and yeasts.

*Mesophilic Microbes.*—Minimum at 5° to 25° C., optimum about 37° C., maximum at about 43° C. To this class belong all pathogenic bacteria, most parasitic and many saprophytic forms.

*Thermophilic Microbes.*—Minimum at 25° to 45° C., optimum at 50° to 55° C., maximum at 60° to 70° C. This class includes a number of soil bacteria which are almost exclusively spore-bearing bacilli. They are also found widely distributed in feces.

By carefully elevating or reducing the temperature the limits within which a species will grow can be altered. Thus, the anthrax bacillus may be gradually made to accommodate itself to a temperature of 42° C., and pigeons, which are comparatively immune to anthrax, partly on account of their high body temperature (42° C.), when inoculated with this anthrax succumb to the infection. Another culture accustomed to a temperature of 12° C. kills frogs kept at 12° C. We have cultivated a very virulent diphtheria bacillus so that it will grow at 43° C. and produce strong toxin.

**Effect of Low Temperature.**—Temperatures even far under 0° C. are only slowly injurious to microörganisms, different species being affected with varying rapidity. This has been demonstrated by numerous experiments in which they have been exposed for weeks in a refrigerating mixture at —18° C. If a culture of typhoid bacilli is frozen, about 50 to 70 per cent. of the organisms are killed at the time. At the end of one week not more than 10 per cent. survive, and at four weeks not over 1 per cent. After six months none survives. More resistant bacteria live longer and spores may survive in ice for years. Bacteria have even been subjected to a temperature of —175° C. by immersing them in liquid air kept in an open tube for two hours, and 15 to 80 per cent. were found still to grow when placed in favorable conditions. We found about 10 per cent. of typhoid bacilli alive after thirty minutes' exposure to this low temperature. Staphylococci were more resistant. Spores were scarcely affected at all.[1]

**Effect of High Temperatures.**—Prolonged temperatures from 5° to 10° C. over the optimum affect microörganisms injuriously in several respects. For instance, varieties may be produced of diminished activity of growth, the virulence of the property of causing fermentation may be decreased, and the power of spore formation may be gradually lost.

If the maximum temperature is exceeded, the organism dies. The thermal death-point for the psychrophilic species is about 37° C., for the mesophilic species about 45° to 55° C., and for the thermophilic species about 75° C. There are no non-sporebearing bacteria, except possibly a few cocci, which when moist are able to withstand a temperature of 100° C. even for a few minutes. A long exposure to temperatures between 60° and 80° C. has the same result as a shorter one at the higher temperatures. Ten to thirty minutes' exposure to moist heat will at 60° C. kill the cholera spirillum, the streptococcus, the typhoid bacillus and the gonococcus and at 70° C. the staphylococcus the latter being among the most resistant of the pathogenic organisms which show no spores. A much shorter exposure will kill a large percentage of any mass of these bacteria.

**Effect of Dry Heat.**—When microörganisms in a desiccated condition are exposed to the action of heated dry air, the temperature required for their destruction is much above that required when they are in a moist condition or when they are exposed to the action of hot water or steam. A large number of pathogenic and non-pathogenic species are able occasionally to resist a temperature of over 100° C. dry heat for from ten minutes to one hour. In any large number of bacteria a few are always more resistant than the majority. A temperature of 120° to 130° C. dry heat maintained for one and a half hours wil destroy all bacteria in the absence of spores. Bacteria freed from surface moisture and preserved in oil are very resistant to heat.

**Resistance of Spores to Heat.**—Spores possess a great power of resistance to both moist and dry heat. Dry heat is comparatively wel borne, many bacterial spores resisting a temperature of over 130° C for as long as three hours. Exposed to 150° C. for one hour, practically all spores are killed. Moist heat at a temperature of 100° C. either boiling water or free-flowing steam, destroys the spores of mos varieties of bacteria within fifteen minutes; certain pathogenic and non-pathogenic species, however, resist this temperature for hours The spores of a bacillus from the soil were destroyed after five and a half or six hours' exposure to streaming steam. They were destroyed however, by exposure for twenty-five minutes in steam at 113° to 116 C. and in two minutes at 127° C. The spores from tetanus bacill may require twenty minutes' exposure to kill them. Spores in fatty media are more resistant to heat, therefore immersed in oil they may be killed with great difficulty.

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[1] See Hilhard and Davis, Jour. Bacteriol., 1918, iii, 423, for bibliography.

The resistance of spores to moist heat is tested by suspending threads, upon which the spores have been dried, in boiling water or steam. The threads are removed from minute to minute and laid upon agar or in broth and kept at a suitable temperature for the germination of any living spores. (See chapter on Disinfection.)

**Influence of Light.**—A large number of microörganisms are inhibited in growth by the action of bright daylight, more are affected by direct sunlight, and when the action of the sun's rays is prolonged they lose their power of developing when later placed in the dark.

Some motile organisms move toward the point of greatest luminosity, others away from it. Light-seeking protozoa have green or yellow chromatophores, and usually at the anterior end a red pigment spot. The violet and blue rays are more active than other parts of the spectrum in determining motion.

The susceptibility of bacteria to light may be tested, according to H. Buchner[1] by suspending a large number of bacteria in nutrient gelatin or agar and pouring the media while still fluid in Petri dishes, upon each of which has been pasted a strip of black paper on the side exposed to the light. The action of heat may be excluded by allowing the ray of light to pass through a layer of water or alum of several centimeters' thickness. After the plates have been exposed to the light for one-half, one, one and a half, two hours, etc., they are taken into a dark room and allowed to stand at 20° to 35° C. a sufficient length of time to allow of growth, and then examined to see whether there are colonies anywhere except on the parts covered by the paper; when the colonies exposed to the light have been completely destroyed, there is lying in a clear sterile field a sharply defined region of the shape of the paper strip crowded with colonies.

Protected by ordinary non-colored glass the sun's rays act very slowly.

Only the ultraviolet, violet and blue rays of the spectrum seem to possess bactericidal action; green light has very much less; red and yellow light, none at all. The action of light is apparently assisted by the admission of air; anaërobic species, like the tetanus bacillus, and facultative anaërobic species, such as the colon bacillus, are able to withstand quite well the action of intense, direct sunlight in the absence of oxygen, for four hours.

According to Richardson and Dieudonné, the mechanism of the action of light may be at least partially explained by the fact that in agar plates exposed to light for a short time (even after ten minutes' exposure to direct sunlight) hydrogen peroxide ($H_2O_2$) is formed. This is demonstrated by exposing an agar plate half-covered with black paper, upon which a weak solution of iodide of starch is poured, and over this again a dilute solution of sulphate of iron; the side exposed to the light turns blue black. In gases containing no oxygen hydrogen peroxide is not produced, and the light has no injurious effect. Access of oxygen also explains the effect which light produces on culture media which have been exposed to the action of sunlight as standing in the sun for a time, when afterward used for inoculation. Some bacteria subsequently introduced into such media grow badly —far worse than in fresh culture media which are kept in the shade.

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[1] Ver. d. deutsch. chem. Gesellsch., xxx, 117, 124 and 1110–1113.

**Influence of Radium.**—*Radio-active fluids* have a slight inhibiting effect on microbal growth, but nothing decided enough to be used for therapeutic purposes has been evolved up to the present time.

**Influence of X-rays.**—These rays have a slight inhibiting effect on microörganisms when they are directly exposed to them.

**Influence of Electricity.**—The majority of the observations heretofore made on this subject would seem to indicate that there is no direct action of the galvanic current on bacteria; but the effect of heat and the electrolytic changes in the culture liquid resulting from the electrolysis may destroy them.

Protozoa may be contracted by moderate induction shocks and killed by strong ones.

When a current of electricity is passed through a liquid medium most active protozoa swim with their long diameters in the direction of the lines of force to assemble behind the cathode. Most flagellates and a few ciliates, however, move toward the anode. The direction of motion has been shown by Dale to vary with the nature and concentration of the medium. This whole question has been little studied.

**Influence of Agitation.**—Meltzer has shown that the vitality o bacteria is destroyed by protracted and violent shaking, which causes a disintegration of the cells. Many species are more quickly autolyzed after violent shaking. Hence shaking is made use of in the production of bacterial vaccines.

**Influence of Pressure.**—Microörganisms in fluids which are subjected to great pressure are for a time inhibited in their growth. Larson found that a direct pressure of 6000 atmospheres kills non-sporebearing bacteria in fourteen hours, and that a pressure of about 12,000 atmospheres for the same length of time is required to kill spore-bearing bacteria. He found that nitrogen under a pressure of 120 atmospheres had no effect upon the organisms tried.[1]

**Osmosis.**—Osmosis, due to differences of pressure between the medium and the microörganisms and to the permeability of the cell membrane for different substances is constantly occurring. Presumably the normal development of an organism takes place when the osmotic pressure within the cell is equal to (isotonic) that of its medium. When an organism is transferred to a new medium with an osmotic pressure markedly different from that of the old one, decided changes in morphology may occur. If the difference is too great or the transfer is too sudden, death may result. If the new medium has a highe pressure, then water is abstracted from the cell and the protoplasm shrinks from its membrane. This is called *plasmolysis*. When the new medium has a lower pressure than the old, the cell may burst. This process is called *plasmoptysis*.

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[1] Larson, Hartzell and Diehl: Jour. Infect. Dis., 1918, xxii, 271.

**Influence of Carbonic Acid under Pressure.**—Some non-sporebearing bacteria are killed by $CO_2$ of 50 atmospheres pressure in about one and a half hours. Others are more resistant. D'Arsonval and Charrin submitted a culture of B. pyocyaneus to a pressure of 50 atmospheres under carbonic acid. At the end of four hours cultures could still be obtained, but the bacillus had lost its power of pigment production. A few colonies were developed after six hours' exposure to this pressure, but after twenty-four hours no development occurred. We have subjected broth and milk containing typhoid, dysentery, diphtheria and colon bacilli to the gas under a pressure of 75 and 150 pounds. Within twenty-four hours 99 per cent. of those in the broth and 98 per cent. of those in the milk were destroyed. Within one week the broth was sterile and within four weeks the milk was sterile. Tubercle bacilli and staphylococci were much more resistant, but little effect was noticed in twenty-four hours. The results were the same whether the cultures were kept at 10° or 25° C. Yeast cells withstand the action of $CO_2$ for more than twenty-four hours.[1] Bottled waters charged with carbonic acid are usually sterile.

**Effect of Drying.** — For growth, microörganisms require much moisture. Want of water affects them in different ways. Upon dried culture media development soon ceases but if aftergrowth has occurred the culture media and organisms are dried quickly. The organisms remain viable for a much longer time than on media dried gradually. The question as to how long the non-sporebearing forms are capable of retaining their vitality when dried on a cover-glass or silk threads has been variously answered. We know now that there are many factors which influence the retention of vitality. Spores, of course, are more resistant than vegetative forms.

The varying results sometimes reported by different observers may be explained by the fact that the conditions under which they were made were different, depending upon the desiccator used, the medium upon which the cultures were grown, and the use of silk threads or cover-glasses. In all these experiments, of course, it should be previously determined that in spore-bearing species there are no spores present. Even when a dried culture lives for a long time the majority of the organisms die in a few hours after drying. We have found 1,500,000 colon bacilli to be reduced to 100,000 after three hours' drying. In tissues or exudates they resist drying much longer than when unprotected.

Encysted protozoa withstand long periods of desiccation. Most forms when dried quickly remain viable much longer than when dried slowly.

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[1] Larson, Hartzell and Diehl: Jour. Infect. Dis., 1918, xxii, 271.

**Duration of Life in Pure Water.**—When microörganisms which require much organic food for their development (and these include most of the pathogenic species) are placed in distilled water, they soon die—that is, within a few days. Their death is largely due to plasmoptysis. Even in sterilized well-water or surface water their life duration does not usually exceed eight to fourteen days, and they rarely multiply. Instances, however, of much more extended life under certain conditions are recorded.

**Tactic Effect of Chemicals.—Chemotaxis.**—The deleterious effect of chemicals, especially those used as germicides, will be considered under Disinfection.

Some chemical substances exert a peculiar attraction for microorganisms, known as *positive chemotaxis*, while others repel them—*negative chemotaxis*. Moreover, all varieties are not affected alike. Oxygen, for example, attracts aërobic and repels anaërobic bacteria, and for each variety there is a definite proportion of oxygen, which most strongly attracts. The chemotactic properties of substances are tested by pushing the open end of a fine capillary tube, filled with the substance to be tested, into the edge of a drop of fluid containing the organisms and examining under the microscope. We are able thus to watch the action of the microbes and note whether they crowd about the tube opening or are repelled from it. Among substances showing positive chemotaxis for nearly all microörganisms are peptone and urea, while among those showing negative chemotaxis are alcohol and many of the metallic salts. Such experiments are, of course, rough. The diffusion of the substances from the tube into the surrounding medium must play an extremely active role in the final result.

## PRODUCTS OF MICROBAL GROWTH.

Microörganisms not only are acted upon by their surroundings, as has already been shown, but they themselves act, often markedly, upon these surroundings. We have spoken, under the Effect of Food (p. 51) of the great changes which may be produced in growths by slight changes in the food medium. So, many of the products, as noted below, are influenced to a greater or a less extent by environment.

**Production of Light.**—Microörganisms which have the property of emitting light (*photogens*) are quite widely distributed in nature, particularly in media rich in salt, as in sea-water. Many of these have been accurately studied. The emission of light is a property of the living protoplasm of the organism, and is not usually due to the oxidation of any photogenic substance given off by them; at least only in two instances has such substance been claimed to have been isolated. While these organisms cannot emit light except during life, they can live without emitting light. They are best grown under free access of oxygen in a culture medium prepared by boiling fish in sea-water (or water containing 3 per cent. sea-salt), to which 1 per cent. peptone, 1 per cent. glycerin, and 0.5 per cent. asparagin are added. The power of emitting light is soon lost unless the organism is frequently transplanted to fresh media.

**Thermic Effects.**—The production of heat by microörganisms does not attract attention in our usual cultures because of its slight amount, and even fermenting culture liquids with abundance of bacteria cause no sensation of warmth when touched by the hand. Careful tests, however, show that heat is produced. The increase of temperature in organic substances when stored in a moist condition, as tobacco, hay, manure, etc., is due, partly at least, to the action of bacteria.

**Chemical Effects.**—The chemical changes which take place in substances as they are split up by microörganisms depend on the nature of the substances involved and the conditions under which they exist, and on the varieties of germs present. Chemists can as yet enumerate only some of the substances evolved and describe in but a few cases the manner in which they were produced. The chemical activity may be divided into the following four types: (1) Production of substances which help in some way the life of the cell or are peculiar to it. These substances may be secreted and retained within the cell, or liberated from it, *e. g.*, ferments or enzymes; true toxins. (2) Production of substances liberated by the bacteria as waste products. (3) Production of substances by the breaking down of the food media, *e. g.*, putrefactive products, due largely to enzyme action. (4) The production of substances which help form the protoplasm of the bacterial cell itself.

**Fermentation.**—Fermentation may be defined broadly as a chemical decomposition of an organic compound, induced by chemical substances elaborated by the organisms as unorganized ferments or enzymes. Fermentation demands the proper nutriment, temperature, moisture, and the absence of deleterious substances. The enzyme itself is not markedly diminished in quantity after the fermentation ceases, though the process yields products that inhibit its action; hence fermentation ceases when the products are in excess, or when the nutriment is exhausted. That the process will often begin again after diluting the fermented medium, shows that the *concentration* of the harmful products plays an important part in the inhibitory action. The fact that the enzyme apparently does not bind itself to any of the end-products allies it to those chemical agents known as "catalysts."

*Characteristics of Enzymes.*[1]—Enzymes are amorphous, non-dialyzable products of living protoplasm. They are soluble in water and in glycerine. They may be precipitated from their solutions either by alcohol or by certain salts, such as ammonium or zinc sulphate. They are easily carried down by different precipitates. They withstand moderate dry heat, but are usually destroyed in watery solutions on exposure of ten to thirty minutes to a temperature of 60° to 70° C. They are sensitive to acids, but are more resistant to alkalies. They, even when present in minute quantities, are able partly to split up or decompose complex organic compounds into simpler substances, and thus to render the foodstuff suitable for microbal growth.

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[1] See discussion of Enzymes in Hawk's Physiological Chemistry, 6th ed., 1918, Phila.

The enzymes may be grouped as sugar-splitting, inverting, fat-splitting, proteoclastic (proteolytic), diastatic, rennin-like or lab enzymes, oxidizing enzymes, and so on.

*Sugar-splitting Enzymes* (*Zymase, Lactase, Maltase, Etc.*).—Many bacteria and yeasts are capable of splitting sugars, especially under anaërobic conditions. The action may be indicated by the following equations:

$$\begin{array}{ccccc} C_6H_{11}O_6 & = & 2C_2H_6O & + & 2CO_2 \\ \text{Grape-sugar.} & & \text{2 Alcohol.} & & \text{2 Carbon dioxide.} \end{array}$$

Or, Or,

$$\begin{array}{ccccc} C_6H_{12}O_6 & = & 2C_3H_6O_3 & = & 3C_2H_4O_2 \\ \text{Grape-sugar.} & & \text{2 Lactic acid.} & & \text{3 Acetic acid.} \end{array}$$

*Fat-splitting Enzymes* (*Lipase*).—Little is known about this enzyme. It has industrial importance because of its action in rendering butter and other fats rancid.

*The Proteoclastic* (*Proteolytic*) *Enzymes*.—The proteoclastic enzymes which are somewhat analogous to trypsin—being capable of changing albuminous bodies into soluble and diffusible substances—are very widely distributed. The liquefaction of gelatin, which is chemically allied to albumin, is due to the presence of a proteölytic ferment, gelase. The production of proteolytic ferments by different cultures of the same variety of bacteria varies considerably—far more than is generally supposed.

Bitter-tasting products of decomposition may be formed by certain liquefying bacteria in media containing protein, as, for example, in milk.

*Diastatic Enzymes*.—Diastatic ferments convert starch into sugar. This action is demonstrated by mixing starch paste with suitable cultures, then adding thymol and keeping the digestion for six to eight hours in the incubating oven; on the addition of Fehling's solution and heating, the reaction for sugar appears—the reddish-yellow precipitate due to the reduction of the copper.

*Inverting Enzymes*.—Inverting ferments (that is, those which convert polysaccharides into monosaccharides) are of very frequent occurrence. Bacterial invertase is slightly less susceptible to heat than are some other ferments, and is produced in culture media free from protein.

*Rennin-like Enzymes*.—Rennin-like ferments (substances having the power of coagulating milk and other liquid proteins with neutral reaction, independent to acids) are found not infrequently among bacteria.

*Oxidizing Enzymes*.—Oxidizing enzymes or oxidases produce change by the addition of oxygen. A common example of this is the production of vinegar chiefly by B. aceti and B. pasteurianum.

*Alkaline Products from Enzyme Action*.—Aërobic bacteria always produce alkaline products from albuminous substances. Many species also produce acids from the breaking up of sugars or related bodies (see below), which explains the fact that neutral or slightly alkaline broth often becomes acid at first from the fermentation of the sugar contained in the meat used for making the media. When the sugar is used up, the reaction often becomes alkaline, as the production of alkalies continues. The substances producing the alkalinity in cultures are chiefly ammonia, and the ammonium bases.

The conversion of urea into carbonate of ammonia by *Micrococcus ureæ* affords an example of the production of alkaline substances by bacteria:

$$\underset{\text{Urea.}}{CO(NH_2)_2} \quad + \quad \underset{\text{2 Water.}}{2H_2O} \quad = \quad \underset{\text{Ammonium carbonate.}}{CO_3(NH_4)_2}$$

**Poisonous Products.**—The poisonous products of microörganisms (the endo- and exotoxins) are considered in Chapter VII.

**Pigment Production.**—Pigments have no known importance in connection with disease, but are of interest and have value in identifying bacteria. Very little is known of their chemical composition. They are of almost every hue. A few only are given here.

**Red and Yellow Pigments.**—Of the twenty-seven red and yellow chromogenic bacteria studied by Schneider, almost all produce pigments soluble in alcohol and insoluble in water. The large majority of these pigments possess in common the property of being colored blue green by sulphuric acid and red or orange by a solution of potash. Though varying considerably in their chemical composition and in their spectra, they may be classified, for the most part, among that large group of pigments common to both the animal and vegetable kingdoms known as *lipochromes*, and to which belong the pigments of fat, yolk of eggs, the carotin of carrots, turnips, etc.

**Violet Pigments.**—Certain bacteria produce violet pigments, also insoluble in water and soluble in alcohol, but insoluble in ether, benzol and chloroform. These are colored yellow when treated in a dry state with sulphuric acid and emerald green with potash solution.

**Blue Pigments.**—Blue pigments, such as the blue pyocyanin produced by B. pyocyaneus; and the fluorescent pigment common to many so-called fluorescent bacteria (bacteriofluorescence) are examples. In cultures the pigment is at first blue; later, as the cultures become alkaline, it is green.

Numerous investigations have been made to determine the cause of the variation in the chromogenic function of bacteria. All conditions which are unfavorable to the growth of the bacteria decrease the production of pigment, as cultivation in unsuitable media or at too low or too high a temperature, etc. The B. prodigiosus seldom makes its red pigment at 37° C., and when transplanted at this temperature, even into favorable media, the power of pigment production is gradually lost. B. pyocyaneus does not produce its blue pigment under anaërobic conditions. Occasionally colored and uncolored colonies of the same species of bacteria may be seen to occur side by side in one plate culture, as, for example, in the case of Staphylococcus pyogenes.

**Reduction Processes.**—The following processes depend wholly or in part upon the reducing action of nascent hydrogen:

1. *Sulphuretted Hydrogen* ($H_2S$).—All bacteria, according to Petri and Maassen, possess the power of forming sulphuretted hydrogen, particularly in liquid culture media containing much peptone (5 to 10 per cent.); only a few bacteria form $H_2S$ in bouillon in the absence of peptone, while about 50 per cent. in media containing 1 per cent. of peptone possess the property of converting sulphur into sulphuretted hydrogen, for which purpose is required the presence of nascent hydrogen. (For demonstration see Chapter IV.)

2. *The Reduction of Blue Litmus Pigments, Methylene Blue and Indigo to Colorless Substances.*—The superficial layer of cultures in contact with the air shows often no reduction, only the deeper layers being affected.

3. *The Reduction of Nitrates to Nitrites, Ammonia and Free Nitrogen.*—The first of these properties seems to pertain to a great many bacteria.

**Aromatic Products of Decomposition.**—Many microbes produce aromatic substances as the result of their growth. The best known of these are indol, skatol, phenol and tyrosin.

**Decomposition of Fats.**—Pure melted butter is not a suitable culture medium for microbes. The rancidity of butter is brought about (1) as the result of a purely chemical decomposition of the butter by the oxygen of the air under the influence of sunlight, and (2) through the formation of lactic acid from the milk-sugar left in the butter. Fats are, however, attacked by bacteria with the consequent production of acid when mixed with gelatin and used as culture media.

**Putrefaction.**—By putrefaction is understood in common parlance every kind of decomposition due to microbes which results in the production of malodorous substances. Scientifically considered, putrefaction depends upon the decomposition of albuminous substances, which are frequently first peptonized and then further decomposed. Typical putrefaction occurs more frequently when oxygen is absent or scanty; the free passage of air through a culture of putrefactive bacteria—an event which does not take place in natural putrefaction—very much modifies the process; first, biologically, as the anaërobic bacteria are inhibited, and then by the action of the oxygen on the products or by-products of the aërobic and facultative anaërobic bacteria.

As putrefactive products we have peptone, ammonia and amines, leucin, tyrosin and other amino substances; oxyfatty acids, indol, skatol, phenol, ptomains, toxins and finally, sulphuretted hydrogen, mercaptans, carbonic acid, hydrogen and possibly marsh gas.

**Nitrifying Bacteria.**—Nitrification is produced by a special group of bacteria, cultivated in the laboratory with difficulty, which do not grow on our usual culture media. From the investigations of Winogradsky it would appear that there are two common microörganisms present in the soil, one of which converts ammonia into nitrites and the other converts nitrites into nitrates.

**Conversion of Nitrous and Nitric Acids into Free Nitrogen.**—This process is performed by a number of bacteria.

The practical importance of these organisms is that by their action large quantities of nitrates in the soil, and especially in manure, may become lost as plant food by being converted into nitrogen.

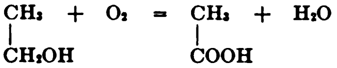
By the aid of certain root bacteria, which gain entrance to the roots of legumes and there produce nodular formations, the leguminous plants are enabled to assimilate nitrogen from the atmosphere. It is not known exactly how this assimilation of nitrogen occurs, but it is assumed that the zoöglea-like bacteria, called *bacteroids*, constantly observed in the nodules, either alone or in a special degree, possess property of assimilating and combining nitrogen. It seems, moreover, to have been recently established that, independently of the assistance of the legumes, certain bacteria exist free in the soil, which accumulate nitrogen by absorbing it from the air. These various nitrifying, denitrifying and nitrogen-fixing bacteria are described in detail in the special chapter upon bacteria in nature.

**Formation of Acids and the Aldehyde Group from Carbohydrates.**—Free acids or hydrogen ions and the aldehyde group are formed by many microbes in culture media containing some form of sugar or other fermentable carbohydrates, such as the alcohol mannite.

Among the acids produced, the most important is lactic acid; also traces of formic acid, acetic acid, propionic acid and butyric acid, and not infrequently some ethyl alcohol and aldehyde or acetone are formed. Occasionally no lactic acid is present.

**Formation of Gas from Carbohydrates and other Fermentable Substances of the Fatty Series.**—The only gas produced in *visible* quantity in sugar-free culture media is nitrogen. If sugar is vigorously decomposed by bacteria, as long as pure lactic acid or acetic acid is produced there may be no development of gas, as, for instance, with the B. typhosus on grape-sugar, but frequently there is much gas developed, especially in the absence of air. About one-third of the acid-producing species also develop gas abundantly, this consisting chiefly of $CO_2$, which is always mixed with H. Marsh-gas is seldom formed by bacteria, with the exception of those decomposing cellulose. (For demonstration see Chapter IV.)

**Formation of Acids from Alcohol and other Organic Acids.**—It has long been known that B. aceti and allied bacteria convert dilute solutions of ethyl alcohol into acetic acid by oxidization:



$$\begin{array}{l} CH_3 \\ | \\ CH_2OH \end{array} + O_2 = \begin{array}{l} CH_3 \\ | \\ COOH \end{array} + H_2O$$

The higher alcohols—glycerin, dulcit, mannite, etc.—are also converted into acids.

Finally, numerous results have been obtained from the conversion of the fatty acids and their salts into other fatty acids by bacteria.

The following general references may also be consulted:

Buschke: Die Sprosspilze in Kolle und Wassermann's Die Mikroörganismen, Jena, 2d edition, 1913.

Calkins: The Protozoa, 1st edition, New York, 1901. Also article entitled The Protozoa, in Osler's Modern Medicine, Philadelphia, 1907, vol. i; also Protozoölogy, New York and Philadelphia, 1909.

Doflein: Lehrbuch der Protozoenkunde, 2d edition, Jena, 1909; Handbuch der pathogenen Mikroörganismen, Kolle und Wassermann, 2d edition, Jena, 1913.

Jennings: Behavior in Lower Organisms, New York, Macmillan & Co., 1906.

Lang: Protozoa in Vergleichende Anatomie der Wirbellosen Thiere, new edition, 1909.

Lankester: Treatise on Zoölogy, 1st edition, London, Part I, first and second fascicles, 1909.

Moore: The Pathology of Infectious Diseases of Animals, 4th edition, New York, 1916.

Oppenheim: Die Fermente. u. ihre Wirkung, Leipzig, 1903.

Petruschky: Die pathogenen Mychomyceten, in Kolle und Wassermann's Die Mikroörganismen, Jena, 2d edition, 1913.

Plaut: Die Hyphenpilze in Kolle und Wassermann's Die Mikroörganismen, Jena, 2d edition, 1913.

# CHAPTER III.

# THE MICROSCOPE AND THE MICROSCOPIC EXAMINATION OF MICROÖRGANISMS.

## THE MICROSCOPE.[1]

If lenses were capable of refracting all light equally, and bringing to a focus in one plane all rays proceeding from one plane in the object the microscope would be a comparatively simple instrument. But simple lenses have several serious optical defects.

1. **Spherical Aberration.**—Points in the plane of the object are imaged on the *curved* surface of the spherical lens. This defect may be somewhat diminished by combining convex and concave lenses, and by restricting the size of the field. Objectives corrected in this way are called *aplanatic.*

2. **Chromatic Aberration.**—This defect is due to the fact that the rays of light vary in their refraction according to their wave length (colors), *e. g.*, the red rays have the longest focus and the violet the shortest. This is considerably corrected by combining planoconcave lenses of flint glass with biconvex lenses of crown glass—*achromatic objectives.*

Still more of a correction is made by combining several different kinds of lenses with a lens of fluorite—*apochromatic objectives.*

Monochromatic light may be employed and thus chromatic aberration may be entirely avoided.

3. **Diffraction.**—Less luminous secondary images about the primary image, due to scratches or foreign particles or other defects may occasionally occur in the very best lenses.

In order to understand fully the principle of the microscope, works on optics should be consulted.

**Different Parts of the Microscope** (Figs 8 and 9).—A complete instrument usually has four oculars, or eye-pieces (*A*) which are numbered from 1 to 4, according to the amount of magnification which they yield. Nos. 2 and 4 are most useful for bacteriological work. The objective—the lens (*B*) at the distal end of the barrel—serves to give the main magnification of the object. For stained bacteria the $\frac{1}{12}$ achromatic oil-immersion lens is regularly employed; for photographic purposes the apochromatic lenses are needed, although even here they are not indispensable. A $\frac{1}{16}$ lens may at times be useful, but hardly necessary; a No. 4 ocular and a $\frac{1}{12}$ lens give a magnification of about 1000 diameters (Fig. 10). For unstained bacteria we employ either the $\frac{1}{12}$ immersion or $\frac{1}{7}$ dry lens, according to the purpose for which we study the bacteria; for the examination of colonies when, as a rule, we do not wish to see individual bacteria but only the general appearance of whole groups, we use lenses of much lower magnification (Fig. 11).

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[1] **Gage: The Microscope, 1917, 12th ed., The Comstock Publishing Co.**

The stage (*C*)—the platform upon which the object rests—should be large enough to support the Petri plates if culture work is to be done. The distance between the optical axis of the instrument and the pillar must be great enough to permit one to examine rather more than half the surface of the Petri dish without revolving it. The iris diaphragm (*D*) opens and closes, and, like the iris of the eye, controls the amount of light. Its opening is diminished or increased by moving a small arm underneath the stage. The reflector or mirror (*E*) placed beneath the stage serves to direct the light to the object to be examined. It has two surfaces—one concave and one plane. The concave surface must not be employed when the substage condenser is being used, otherwise the rays of light reaching the stage from the condenser will not be correctly focussed. The concave surface may be used when unstained

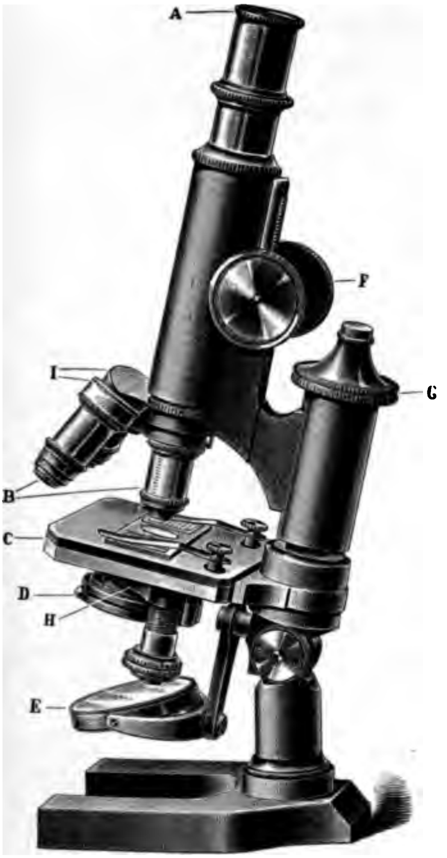

FIG. 8.—Microscope.

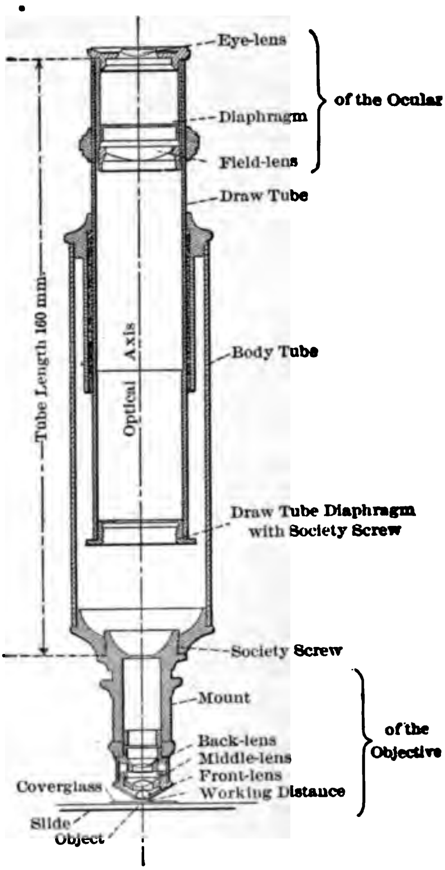

FIG. 9.—Internal structure of the microscope.

objects, such as colonies, or hanging drops are examined. At the same time the Abbé condenser should be lowered and the iris blender (*D*) regulated. The coarse adjustment (*F*) is the rack-and-pinion arrangement by which the barrel of the microscope can be quickly raised or lowered. It is used to bring the bacteria roughly into focus. If the bearings become loose, tighten the little screws at the back of the pinion box. Keep the teeth clean. If the bearings need oiling, use an acid-free lubricant, such as paraffin oil. The fine adjustment (*G*) serves to raise and lower the barrel very slowly and evenly, and is used for the exact study of the bacteria when high-power lenses are used. It is necessarily of limited range and delicate in its mechanism. If, when looking into the eye-piece, no change of focus is noticed by turning the micrometer head, or if the micrometer head ceases to turn, the adjustment has reached its limit. Raise the barrel of the microscope by means of the coarse adjustment, then turn the micrometer back to bring the fine adjustment midway within its range. When the fine adjustment head stops, do not force it. For the microscopic study of microbes it is essential that we magnify the organisms as much as possible and still have their definition clear and sharp. For this purpose the microscope should be provided with an oil-immersion system and a substage condensing apparatus. In using the oil-immersion lens a drop of oil (oil of cedar) of the same index of refraction as the glass is placed upon the face of the lens, to connect it with the coverglass when the bacteria are in focus. There is thus no loss of light through deflection, as is the case in the dry system. If the lenses become dirty, they should be wiped gently with Japanese lens paper or a clean, soft, old-linen handkerchief. If necessary, breathe on the lens before wiping, and if this does not succeed, use a little xylol or chloroform. These substances are not to be used unless necessary. An immersion objective should always be cleaned immediately after

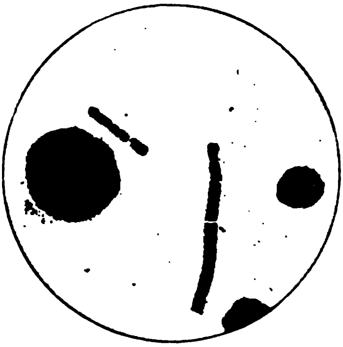

FIG. 10.—Anthrax bacilli and blood cells. × 1000 diameters.

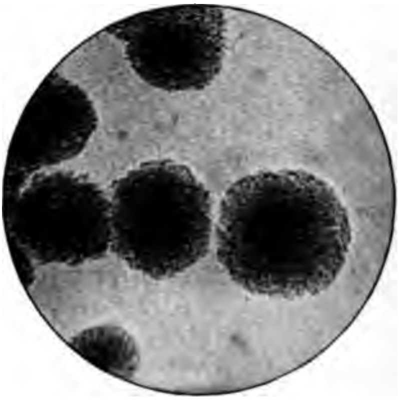

FIG. 11.—Colonies of diphtheria bacilli. × 200 diameters.

using. The objective should always be kept covered so as to prevent dust dropping in.

**Light.**—The best light is obtained from white clouds or a blue sky with a northern exposure. Avoid direct sunlight. If necessary, use white shades to modify the sunlight. Artificial light has one advantage over daylight in that it is constant in quality and quantity. The Welsbach burner and a whitened incandescent bulb give a good light. A blue glass between the artificial light and the lens is often of value. An eye shade may be helpful.

**Substage Condensing Apparatus** (*H*) is a system of lenses situated beneath the central opening of the stage. It serves to condense the light passing from the reflector to the object in such a way that it is focussed upon the object, thus furnishing the greatest amount of luminosity. Between the condenser and the reflector is placed the iris diaphragm.

**Focussing.**—This is only difficult with the higher magnifications. Focus the body tube down by means of the coarse adjustment until the objective approaches very near to the cover-glass, being careful not to touch it. Observe from the side where the tip of the objective casts a shadow on the cover-glass. Then with the eye at the eye-piece focus up carefully with the coarse adjustment until the specimen comes plainly into view. Be careful not to pass this focal point. It is easily unnoticed if the light is too intense and the specimen thin and transparent. If the sliding-tube coarse adjustment is used, focus carefully by giving the tube a spiral movement.

When the object is brought fairly well into focus by means of the coarse adjustment, use the fine adjustment to focus on the particular spot desired, for if this spot is in the center of the field of the low power it should be somewhere in the field of the higher power. It is too much to ask of the maker that the lenses be made absolutely par-focal and centered. The delicacy of the centering can be appreciated when the magnification and the extremely small portion examined are considered. When the objectives are not thus fitted to the nose-piece, refocussing and again hunting up the object are necessary. In so doing we repeat the caution always to focus up before turning the nose-piece. When no revolving nose-piece is used, the change of objectives means the unscrewing of one and the screwing of the other into its place and refocussing.

The beginner should always use the low-power objectives and oculars first. The low-power objectives have longer working distances and are not so apt to be injured. They always show a larger portion of the specimen and thus give one a better idea of the general contour. After obtaining this general idea the higher powers can be used to bring out greater detail in any particular part. Generally speaking, it is best to use a high-power objective and low-power eye-piece in preference to a low-power objective and high-power eye-piece. In the latter case any imperfections in the objective are magnified unduly by the eye-piece, giving, as a rule, poor definition.

**Tube Length and Cover-glass.**—All objectives are corrected to a certain tube length (160 mm. by most makers—Leitz, 170 mm.) and all objectives in fixed amounts of over 0.7 N. A. are corrected to a definite thickness of cover-glass as well (Zeiss, 0.15 mm., 0.2 mm.; Leitz, 0.17 mm.; Bausch & Lomb and Spencer, 0.18 mm.). These objectives give their best results only when used with the cover-glass and tube length for which they are corrected. As indicated in Fig. 9 the tube length extends from the eye-lens of the eye-piece to the end of the tube into which the objective or nose-piece is screwed. If a nose-piece is used, the draw tube must be correspondingly shortened. If the cover-glass is thinner than that for which the objective is corrected, the tube must be lengthened to obtain best results; if thicker, shortened.

The more expensive objectives are provided with adjustable mounts by which the distances between the lens systems may be changed to compensate for difference of thickness of cover. They are successfully used only in the hands of an expert. One of them out of adjustment is worse than an ordinary objective.

**Dark-ground Illumination and the Examination of Ultramicroscopic Particles.**—The apparatus constructed by Siedentopf and Zsigmondy makes visible, even in solutions otherwise apparently homogeneous, very minute particles, which heretofore could not be seen even with the highest magnifications. Particles $1\mu\mu$ (a millimicron = one-millionth of a millimeter) are thus rendered visible.

This increased power in microscopic analysis is made possible by intense (electric arc lamp) lateral focal illumination of the objects examined, and by shutting off the rays passing in the usual way through the tube of the microscope. The greater the difference between the refractive index of the objects colloidally dissolved or otherwise held in suspension and the fluid which surrounds them, the brighter will be the appearance of the objects, and therefore the more readily visible.

The microscope field is dark; the objects which refract the light show as brightly illuminated, sharply defined pictures, in which the black margin corresponds to the contour of the object.

Reichert,[1] of Vienna, has simplified this apparatus by devising another condenser. The light which illuminates the object has a greater refraction than the cone of light entering the objective which produces the image.

With this apparatus such living organisms as the *Treponema pallidum* and the flagella on certain bacteria, which can scarcely be seen by ordinary microscopes on account of their low refractive indices, may be demonstrated with great clearness.

The use of *microphotography with ultraviolet light* (according to A. Kohler[2]) makes visible particles that cannot be seen by ordinary light, because of the inability of the violet rays to pass through certain substances, *e. g.*, chromatin.

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[1] Jour. Royal Micro. Soc., 1907, p. 364.

[2] Ztschr. f. wiss. Mikros., 1904, xxi, 129.

This method is said to increase 40,000 times our present limit of vision. The few discoveries claimed by these means for diseases of unknown origin have so far lacked sufficient corroboration to constitute them proved.

## MICROSCOPIC METHODS.

The direct microscopic examination of suspected substances for microorganisms can be made either with or without staining. Unstained, the microbes are examined living in a hanging drop or on transparent solid media, under daylight, or better, artificial light, to note their number, their motility, their size, form and spore formation, their general arrangement, their reactions to specific serums and to vital stains, etc. But for more exact study of their structure they must be stained in a dried film preparation on a glass slide or a cover-glass or when in tissues, in sections.

**Elimination of Foreign Organisms from Preparations.**—Since germs are present in the air, in dust, in tap-water, on our bodies, clothes and on all surrounding objects, it follows that when we begin to examine substances for microbes the first requisite is, that the materials we use, such as staining fluids, cover-glasses, etc , should be practically free from organisms, both living and dead, otherwise we may not be able to tell whether those we detect belong originally in the substances examined or only in the materials we have used in the investigation. Therefore, all solutions are filtered and all apparatus thoroughly cleaned and when necessary sterilized.

**Examination of Bacteria in the Hanging Drop.**—For this examination special slides and methods are desirable. The slide used is one in which there is ground out on one surface a hollow having a diameter of about ½ inch (Fig. 12). According to the purpose for which the hanging drop is to be studied, sterilization of the slide and cover-glass may or may not be necessary.

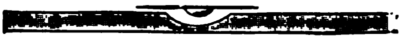

FIG. 12.—Hollow slide with cover-glass.

The technic of preparing and studying the hanging drop is as follows: The surface of the glass around the hollow in the slide is smeared with a little vaselin or other inert oil. This has for its purpose both the sticking of the cover-glass to the slide and the prevention of evaporation in the drop placed in the little chamber, which is to be formed between the cover-glass when placed over the hollow, and the slide.

If the bacteria to be studied are in a fluid, we place a platinum loopful upon the center of the cover-glass and, to avoid drying, immediately invert it by means of a slender pair of forceps over the hollow in the slide, being very careful to have the drop over the center of the cover-glass. The cover-glass is then pressed on the slide so as to spread the vaselin and make a perfect seal. If the bacteria are growing on solid media, or are obtained from thick pus or tissues from organs, they are mixed with a suitable amount of bouillon or sterile physiological salt solution[1] either before or after being placed upon the conver-glass.

In studying living bacteria to determine only their grouping and motion we may use less magnification than for studying other characteristics. In studying unstained bacteria and tissues we shut off as large a portion of the light with our diaphragm as is compatible with distinct vision, and thus favor contrasts which appear as lights and shadows, due to the differences in light transmission of the different materials under examination. It is necessary to remember that they are seen with difficulty, and that we are very apt, unless extremely careful in focussing, to allow the lens to go too far, and so come upon the cover-glass, break it, destroy our preparation, and, if examining pathogenic bacteria, infect the lens. This may be avoided by first finding the hanging drop with a low-power lens and then centering it. The edge of the drop is focussed more easily than the center. The lens of higher magnification is now very gradually lowered, while watched from the side until the tip of the objective throws a shadow on the cover-glass. Then with eye to eye-piece focus up carefully until organisms come into view.

**Hanging Mass or Hanging Block Cultures.**—In order to study the morphology and manner of multiplication of individual microörganisms to better advantage than in the hanging drop, we have used hanging masses of agar, made by placing a large platinum loopful of melted agar on a sterile cover-glass and allowing it to harden, protected from dust. The organisms are placed on the free surface of this mass which is then inverted over a hollow slide and studied as in a hanging drop.

Hill devised the following procedure: Melted nutrient agar is poured into a Petri dish to a depth of about ⅛ to ¼ inch. When cool, a block is cut out about ¼ inch square. The block is placed, under surface down, on a slide and protected from dust. A very dilute suspension of the growth to be examined is then made in sterile bouillon and spread over the upper surface of the block. The slide and block are then put in the incubator for ten minutes to dry slightly. A clean cover-slip is now placed on the agar block in such a way as to avoid large air bubbles. The slide is then removed. With the aid of a platinum loop a drop or two of melted agar is run along each side of the block to fill an angles between it and the cover-glass. After drying in the incubator for five minutes it is placed over a hollow slide and sealed with paraffin.

We consider the hanging-mass method better than that of the hanging block in many instances, because in the former method no pressure is exerted on the bacteria, and more oxygen is allowed them.

**Film Preparation** (Spread, Smear).—Film preparation is made as follows: A very small amount of the blood, pus, discharges from mucous membranes, cultures from fluid media, or other material to be examined is removed, usually by means of a sterile swab or platinum loop, and smeared undiluted in an even, thin film over a perfectly clean,[1] thin cover-glass or slide. From cultures on solid media, however, on account of the abundance of organisms in the material, a little of the growth is diluted by adding it to a small loopful of filtered or distilled water, which has been previously placed on the glass slide. It is best to add to the drop just enough of the culture to make a perceptible cloudiness. Blood films may be made either by the cover-glass or the slide method.

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[1] Physiological salt solution is usually 0.8 per cent. NaCl in distilled water.

To make a cover-glass preparation, two square, very thin (hence flexible) cover-glasses are cleaned. Holding one with thumb and index fingers by opposite corners, the tip of a drop of blood obtained by needle puncture of finger or lobe of ear is made to touch the center of the cover-glass, and the second clean cover-glass held similarly is allowed to fall upon the first one in such a manner that the corners do not coincide. The blood droplet spreads by capillarity into a thin film, which is a sign to pull the two covers apart in the plane in which they lie; good results depend upon cleanliness, rapidity and success in sliding the two covers apart.

To make a slide film, the tip of the exuded blood drop is made to touch one slide near one end, and the edge of the second slide, held at an acute angle to the first one, is made to bisect the drop, which will spread at the point of contact by capillarity across the slide. Upon pulling the second or spreading slide over the first slide, never changing the angle and applying gentle pressure, a thin layer of blood suitable for examination will be formed. A slide made in this manner should be dried immediately by agitation in the air. It may then be fixed and stained in various ways.

Milk films, after fixation, are cleared of fat by means of ether, xylol or alkaline solution.[2]

The film either is dried thoroughly in the air and then fixed with heat or any chemical fixative, or it may be placed in any of the fixatives while still moist. The usual fixatives are methyl alcohol, absolute ethyl alcohol, Zenker's solution, etc. (see pp. 86 and 87).

When fixed with heat, the glass is held by any one of the several kinds of forceps commonly used; and is passed three times by a rather slow movement through the Bunsen or alcohol flame.

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[1] To render new cover-slips clean and free from grease, the method recommended by Gage is useful: Place in following solution overnight:

| | |
|---|---|
| Bichromate of potash ($K_2Cr_2O_7$) | 200 gm. |
| Water, tap or distilled | 800 c.c. |
| Sulphuric acid | 1200 c.c. |

The bichromate is dissolved in the water by heating in an agate kettle; the sulphuric acid is added very slowly and carefully on account of great heat developed. After cooling, it is kept in a glass vessel. It may be used more than once.

Glasses are removed the next morning and cleansed in running tap-water until the yellow color disappears. They are then placed in ammonia alcohol until used. When used, wipe with soft, clean linen or cotton cloth. If old cover-slips are used, boil first in 5 per cent. sodium carbonate solution.

Another procedure is, after washing with soap and water and rinsing in water, to soak the cover-glasses in alcohol, then wipe with soft linen, then place in a Petri dish, and heat in the dry sterilizer for one hour at 200° C. to burn off fatty substances. The heating may be done by holding the cover-glass in the flame sufficiently to heat thoroughly without softening. A cover-glass is not clean when a drop of water spread over it does not remain evenly distributed, but gathers in droplets.

[2] One-half or 1 per cent. sodium hydrate.

The film thus prepared is usually stained either by the simple addition of a solution of an aniline dye, for from a few seconds to five minutes, or by one of the more complicated special stains described later. When the stain is to be hastened or made more intense, the dye is used warm.

The cover-glass or slide, with the charged side uppermost, may either rest on the table or be held by some modification of Cornet's forceps. When the solution is to be warmed, the cover-glass may be floated, smeared side down, upon the fluid contained in a porcelain dish resting on a wire mat, supported on a stand; or the solution may be poured on the glass which may then be held over the flame in the Cornet forceps. If a slide is used, it is simply inserted in the fluid or covered by it. The fluid both in the dish and on the slide should be carefully warmed so as to steam without actually boiling. The slide should be kept completely covered with fluid.

After staining the film, the cover-glass or slide is grasped in the forceps and thoroughly but gently washed in clean water and then dried, first between layers of filter paper and then in the air or high over a flame. If a cover-glass has been used, a drop of balsam or water is replaced on a glass slide and the cover-glass put upon it with the film side down. Films made on slides are usually unmounted. Cedar oil is added at the time of examination with the oil-immersion lens and washed off with xylol immediately after.

*Burri's India-ink Method of Demonstrating Bacteria.*[1]—In 1907, 1908 and 1909 Burri recommended the following method for isolating and studying single bacterial cells: A solution of India ink (flüssige Perltusche) in water 1 to 10 (better 1 to 4) is sterilized in test-tubes in the autoclave for fifteen minutes. A small drop of this ink is mixed carefully with a drop of the fluid to be examined. If cultures from isolated cells are desired, the fluid should first be diluted so that a drop contains *presumably* a single organism; then drops of the mixture are placed in rows upon nutrient agar plates. If the bacteria are to be examined immediately, a drop of the mixture (ink plus undiluted bacterial fluid) is allowed to dry upon a glass slide and then examined under an oil-immersion lens. The bacteria appear a brilliant white upon a dark field, particles of the ink surrounding the organisms like a capsule. This method is especially applicable for the demonstration of such organisms as the Tr. pallidum which have poor staining qualities and a low index of refraction.

**Stains and Staining Methods for Microörganisms.**—Protozoa stain in general as do animal cells. The protoplasm of mature bacteria reacts to stains much as does the nuclear chromatin of animal cells, though the intensity of the staining varies somewhat with the condition of growth, such as the age, the species, the media and so on.

The best bacterial stains are the basic anilin dyes, which are compounds derived from the coal-tar product anilin ($C_6H_5NH_2$).[2]

**Anilin Dyes**—The anilin dyes which are employed for staining purposes are divided into two groups. In one the basic part of the molecule acts and the stains are spoken of as nuclear stains, since they color the nuclear chromatin of both cells and bacteria. In the other the staining act depends upon the acid part of the molecule, and the bacteria and cytoplasm of the higher cells are stained faintly. The stains in the latter group are used chiefly for contrast coloring. The basic dyes are usually employed as salts of hydrochloric acid, while the acid dyes occur as sodium or potassium salts.

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[1] Das Tuscheverfahren als einfaches Mittel, etc., Jena, 1909.

[2] For a good description of the composition and action of the various stains, see A. B. Lee's Microtomist's Vade-Mecum, 7th edition, 1913.

The following are the most commonly used basic anilin stains:

Blue stains—methylene blue, thionin blue } (give the best differentiation;
Red stains—basic fuchsin, safranin } difficult to overstain).
Brown stain—Bismarck brown (weak, may be used as counter-stain).
Green stain—methyl green.
Pink stain—eosin (weak; may be used as counter-stain).
Violet stains—methyl violet, gentian violet, crystal violet (most intense stains; may overstain).

These dyes are all more or less crystalline powders, and while some are definite chemical compounds, others are mixtures. For this reason various brands are met with on the market, and the exact duplication of stains is not always possible. Dyes should be obtained from reliable houses only.

It is advisable to keep on hand *stock saturated alcoholic solutions* from which the staining solutions are made. These stock solutions are made by pouring into a bottle enough of the dye in substance to fill it to about one-quarter of its capacity. The bottle should then be filled with 95 per cent. ethyl alcohol, tightly corked, well shaken, and allowed to stand twenty-four hours. If at the end of this time all the staining material has been dissolved, more should be added, the bottle being again shaken and allowed to stand for another twenty-four hours. This must be repeated until a permanent sediment of undissolved coloring matter is seen upon the bottom of the bottle. This bottle will then be labelled "saturated alcoholic solution," of whatever dye has been employed. The dilution for use in staining is made by filling a small bottle three-fourths with distilled water, and then adding the concentrated alcoholic solution of the dye, little by little, until one can just see through the solution. It is sometimes desirable to use a more concentrated solution with dyes such as methylene blue. Care must be taken that the color does not become too dense; usually about 1 part to 10 is sufficient.

**General Observations on the Principles of Staining Microörganisms.**—The staining of microbes is not to be considered simply as a mechanical saturation of the cell body with the dye, in which the latter is dissolved in the plasma. It is supposed that a chemical combination between the dye substance and parts of the organism may also occur.

The dependence of the staining process upon the solvent condition of the dye is shown in the following observations:

1. Entirely water-free, pure alcoholic dye solutions do not stain well.
2. Absolute alcohol does not decolorize bacteria, while diluted alcohol is an active decolorizing agent. The compound of dye substance and plasma is therefore insoluble in pure alcohol.
3. The more completely a dye is dissolved the weaker is its staining power. For this reason pure alcoholic solutions are inactive; and the so-called weak dye solutions to which strong dye solvents have been added are limited in their action on certain bacteria in which the dye substance is closely united. This is the principle of Neisser's stain[1] for diphtheria bacilli, viz., acetic-acid-methylene-blue solution.

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[1] Ztschr. f. Hyg., 1897, xxiv, 443.

On the other hand, the addition of alkalis to the dye mixture renders the solvent action less complete and the staining power more intense. According to Michaels, however, in Löffler's methylene-blue solution the role of the alkali is purely of a chemical nature, by which it converts the methylene blue into methylene azur (azur II).

The dependence of the staining process upon the nature of the microbe is exhibited in the following facts:

Certain microbes stain easily, others with difficulty. To the latter belong, for example, the tubercle bacillus and lepra bacillus. Spores and flagella also stain with difficulty. The easily stained objects require but a minimum of time to be immersed in a water solution, while the others must be stained by special dyes with or without the aid of outside influences (heat, mordants, etc.). The difficultly stained objects are at the same time not easily decolorized. The explanation of the resistance which these bacteria show to staining as well as to decolorizing agents is to be sought in two ways: either on the assumption that they possess a difficultly permeable or a resisting envelope, or that they have a special chemical constitution. The latter hypothesis holds good only, if at all, in regard to flagella and spores; while the assumption of the resisting envelope has reference more particularly to the tubercle bacillus, and is probably correct. The presence of fatty and waxy bodies in the envelope of these microörganisms is capable of demonstration. Moreover, after extraction of these bodies by ether the tubercle bacillus loses its power of resisting acids, which peculiar resistance can also be artificially produced in other bacteria having normally no such resisting power. In many instances, doubtless, both of these causes, viz., resistant envelope and chemically different constitution, work together to produce the above-mentioned results.

Elective staining properties, whereby certain species of organisms are exclusively or rapidly and intensely stained by certain dyes, have repeatedly been observed. Of the greatest practical importance in this respect is the *Gram stain* (see p. 79 and Chapter VI), used for the differential diagnosis of many species of bacteria; although a distinct classification of bacteria into those which are stained and those which are not stained by Gram's solution has been shown to be impracticable. There are some bacteria, however, which act uniformly toward Gram under most conditions; as, for example, the anthrax bacillus and the pyogenic cocci are always positive, the cholera and plague bacilli and gonococci are always negative to Gram. Other species, again, are at one time stained and at another decolorized by Gram; thus pyocyaneus is stained only in young individuals. Previous heating or extraction with ether does not prevent the action of Gram's stain, but treatment with acids or alkalies renders it impossible. Bacteria so treated, however, after one hour's immersion in Löffler's mordant regain their property of staining with Gram.

As to the nature of Gram's staining solution, it may be mentioned that only the pararosanilines (gentian violet, methyl violet and Victoria blue) are suitable for the purpose, whereas the rosanilines (fuchsin and methylene blue) give negative results. The reason for this is that the iodin compounds with the pararosanilines are fast colors, while those with the rosanilines are unstable. These latter compounds when treated with alcohol break up into their constituents, the iodin is washed out and the dye substance remaining in the tissues stain them uniformly; that is, without differentiation. But iodin-pararosaniline compounds are not thus broken up and consequently stain those portions of the tissue more or less, according to the affinity which they have for the dye substance. The parts stained by Gram are thus distinguished from those stained violet, not only quantitatively, but qualitatively; it is not a gentian violet, but an iodin-pararosaniline staining which occurs.

**Mordants and Decolorizing Agents.**—We have already noted that the protoplasm of unrelated microbes may respond differently to the several dyes. There is, however, seldom any difficulty in selecting a dye which will stain sufficiently to make microbal cells in pure cultures distinctly visible. When the microbes are imbedded in tissue or mixed in a film with blood or pus, it is frequently difficult to prevent the stain from so acting on the tissue or pus elements as to obscure the organisms. Various methods are then employed to stain the germs more intensely than the tissues or to decolorize the tissue more than the organisms. Heating, the addition of alkali to the staining fluid and prolonging the action of the dyes increase the staining properties. We regulate these so as to give the best results. We also use mordants; that is, substances which fix the dye (whether physically or chemically is still to be decided) to the bacterial cell, such as aniline oil or solutions of carbolic acid in metallic salts. The decolorizing agents used chiefly are mineral acids, vegetable acids, diluted alcohols, various oils and hot water.

**Formulæ of the More Generally Used Staining Mixtures.**—LÖFFLER'S METHYLENE-BLUE SOLUTION.—Saturated alcoholic solution of methylene blue, 30 c.c.; caustic potash in a 0.01 per cent. solution, 100 c.c. (The alkali not only makes the cell more permeable, but also increases the staining power by liberating the free base from the dye.)

Films stained two to five minutes, heated if more intense stain is desired. Sections stained one-quarter to several hours and decolorized until faint blue; contrast stain eosin; washed, dehydrated, cleared and mounted (see p. 85).

CARBOLFUCHSIN, OR ZIEHL-NEELSEN SOLUTION.—Distilled water, 100 c.c.; carbolic acid (crystalline), 5 gm.; alcohol, 10 c.c.; fuchsin, 1 gm.; or it may be prepared by adding to 100 c.c. of a 5 per cent. watery solution of carbolic acid, 10 c.c. of a saturated alcoholic solution of fuchsin. The carbolic acid, like the alkali, favors the penetration of the stain.

The last two methods, combined with heating, are used to stain spores and certain resistant bacteria as the tubercle bacilli and other "acid resisters," so that they retain their color when exposed to decolorizing agents (see below).

*Carbol gentian Violet.*—One part of saturated alcoholic solution of gentian violet to 10 parts of a 2 to 5 per cent. solution of carbolic acid.

*Carbol-methylene blue*, first used by Kühne, consists of 1.5 gm. of methylene blue, 10 gm. of absolute alcohol, and 100 c.c. of a 5 per cent. solution of carbolic acid. *Carbolthionin* consists of 10 parts of a saturated alcoholic solution of thionin and 100 parts of a 1 per cent. solution of carbolic acid.

POLYCHROME METHYLENE BLUE (GOLDHORN).—To prepare the stain dissolve 1 gm. lithium carbonate in 200 c.c. clean water and add 1 gm. methylene blue. Shake and dissolve. Pour into porcelain dish over water-bath, stirring frequently until blue color changes to a rich purple. Run through cotton in funnel; make up to 200 c.c. To 100 c.c. add 5 per cent. acetic acid until a faint pink is just visible on litmus paper above level of point discolored by the dye. Now add the remaining 100 c.c. of dye and allow to stand in open dish for forty-eight hours. Run once more through cotton into clean bottle.

It is not necessary to use distilled water, and satisfactory results are obtained with all the different forms of methylene blue tried. B-X (Grübler) is preferable.

Fix the smear by immersion in commercial wood alcohol for fifteen to thirty seconds; wash well and stain for about ten to fifteen seconds in polychrome; wash and stain for from fifteen to sixty seconds in 0.05 per cent. aqueous eosin. Wash again in water and dry in air without heat. Body of parasites blue; chromatin is red to purple.

Results may be varied by using polychrome or eosin for different lengths of time. Admirable preparations may be obtained, even when there is precipitation, by just rinsing the smear a little in 50 per cent. ethyl alcohol. This will remove any precipitation.

Koch-Ehrlich Anilin Water Solution of Fuchsin or Gentian Violet is prepared as follows: To 98 c.c. of distilled water add 2 c.c. anilin oil, or more roughly but with equally good results, pour a few cubic centimeters of saturated anilin oil into a test-tube, then add sufficient water nearly to fill it. In either case the mixtures are thoroughly shaken and then filtered into a beaker through moistened filter paper until the filtrate is perfectly clear. To 75 c.c. of the filtrate (anilin water) add 25 c.c. of the saturated alcoholic solution of either fuchsin, methylene blue, or gentian violet, or add the alcoholic solution until the anilin water becomes opaque and a film begins to form on the surface.

Gram's Stain.—Another differential method of staining which is employed is that known as Gram's method. In this method the object to be stained is floated on or covered with the anilin or carbolic gentian-violet solution described above. After remaining in this for a few minutes it is rinsed in water and then immersed in an iodin solution (Lugol's), composed of iodin, 1 gm.; potassium iodide, 2 gm.; distilled water, 300 c.c. In this it remains for from one to three minutes and is again rinsed in water. It is then placed in strong alcohol until most of the dye has been washed out. If the cover-glass as a whole still shows a violet color, it is again treated with the iodin solution, followed by alcohol, and this is continued until no trace of violet color is visible to the naked eye. It may then be washed in water and examined, or before examination it may be counter-stained for a few minutes by a weak solution of a contrasting dye, such as eosin, fuchsin, carmin or Bismarck brown. This method is useful in demonstrating the capsule which is seen to surround some bacteria—particularly the pneumococcus—and also in differentiating between varieties of bacteria; for some do and others do not retain their stain when put in the iodin solution for a suitable time (see Chapter VI for further remarks upon Gram's stain; see also p. 77).

*The Modifications of Gram's Stain are Many.*—One only is given here.

*Nicolle's Modification.*—Stain cold in carbol-gentian violet one minute; wash in tap-water; stain cold in the iodin mixture one minute; wash in tap-water; decolorize ten seconds in acetone (1 part) and alcohol (3 parts); wash in tap-water; counter-stain ten seconds in dilute carbolfuchsin (1 to 10).

*Stain for B. Diphtheriæ.*—A good polychrome methylene blue solution, *e. g.*, Löffler's methylene blue, brings out the granules perhaps more differentially than any other stain.

Stain 1.

| | |
|---|---|
| Gentian violet (saturated alcoholic solution) | 10.00 c.c. |
| Acetic acid solution, 4½ per cent. (distilled water being used) | 90.00 c.c. |

Stain 2.

| | |
|---|---|
| Bismarck brown | 0.48 mg. |
| Distilled water | 125.00 c.c. |

*Beck's Method.*[1]—The water should be heated to boiling, then the Bismarck brown should be added, and the mixture boiled for one or two minutes and filtered when cool.

The specimen should be stained for thirty seconds with Stain 1, the excess stain washed off with water, and without the specimen's being dried Stain 2 should be added for ten or fifteen seconds, and the specimen washed in running water, dried and examined with oil immersion.

The bacillus, when present, stains as follows: The body of the bacillus becomes a delicate shade of purple or brown, and the clubbed ends and polar bodies, dark purple or black.

*Neisser Stain.*—The Neisser stain is carried out by placing the cover-slip smear of diphtheria or other bacilli in solution No. 1 for from two to three seconds, and then, after washing, in No. 2 for from three to five seconds. The bacilli will then appear either entirely brown or will show at one or both ends a dark blue, round body. With characteristic diphtheria bacilli, taken from a twelve to eighteen hours' growth on serum, nearly all will show the blue bodies (Fig. 116), while with pseudo types (Fig. 122), few will be seen.

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[1] Jour. Amer. Med. Assn., 1918, lxxi, 109.

The solutions are as follows:

No. 1.

| | |
|---|---|
| Alcohol (96 per cent.) | 20 parts |
| Methylene blue (Grübler) | 1 part |
| Distilled water | 950 parts |
| Acetic acid (glacial) | 50 parts |

No. 2.

| | |
|---|---|
| Bismarck brown | 1 part |
| Boiling distilled water | 500 parts |

*Stain for B. Pertussis.*—Toluidin blue, 5 grams; alcohol, 100 c.c.; water, 500 c.c.

**Staining of Capsules.**—Many methods of demonstrating the capsule have been devised. Three only will be given here.

WELCH'S GLACIAL ACETIC ACID METHOD is as follows: (1) Cover the preparation with glacial acetic acid for a few seconds; (2) drain off and replace with anilin gentian-violet solution; this is to be repeatedly added until all the acid is replaced; (3) wash in 1 or 2 per cent. solution of sodium chloride and mount in the same. Do not use water at any stage. The capsule stains a pale violet. (See Plate III, Fig. 15.)

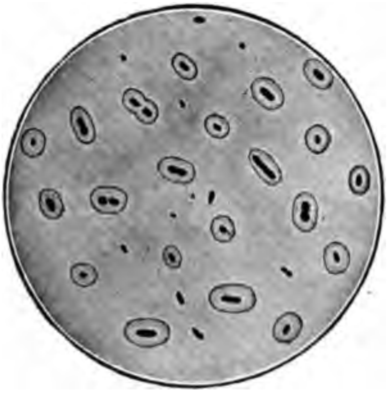

FIG. 13.—Capsule stain by Hiss's method. Rhinoscleroma bacillus. × 1000. (Thro.)

HISS'S COPPER SULPHATE METHOD (Fig. 13).[1]—The organisms are grown, if possible, on ascitic fluid or serum media. If not, the organisms should be spread on the cover-glass mixed with a drop of serum, or better, with a drop of one of the diluted serum media. Dry in the air and fix by heat.

The capsules are stained as follows: A 5 per cent. or 10 per cent. aqueous solution of gentian violet or fuchsin (5 c.c. saturated alcoholic solution gentian violet to 95 c.c. distilled water) is used. This is placed on the dried and fixed cover-glass preparation and gently heated for a few seconds until steam arises. The dye is washed off with a 20 per cent. solution of copper sulphate (crystals). The preparation is then placed between filter paper and thoroughly dried. (See Plate III, Figs. 14 and 16.)

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[1] Jour. Exper. Med., 1905, vi, 317.

HUNTOON'S METHOD[1] (Fig. 1, p. 33).—A 3 per cent. solution in distilled water of nutrose (sodium casinate) is cooked for one hour in an Arnold and tubed unfiltered after adding 0.5 per cent. carbolic. The organisms to be stained are mixed with a drop of this solution of nutrose, spread in thin film on glass slide and dried in air, not fixed. The stain is as follows:

| | |
|---|---|
| Carbolic acid (2 per cent. aqueous solution) . . . . . . | 100.0 c.c. |
| Acetic acid (1 per cent. aqueous solution) . . . . . . | 1.0 " |
| Lactic acid (concentrated) . . . . . . . . . . . . | 0.5 " |
| Carbol fuchsin . . . . . . . . . . . . . . . | 1.0 " |
| Basic fuchsin (saturated alcoholic solution) . . . . . . | 1.0 " |

The stain is kept on the film for thirty seconds, washed in water and dried.

**Staining Spores.**—We have already noted that during certain stages in the growth of a number of bacteria, spores are formed which refuse to take up color when the bacteria are stained in the ordinary manner. Special methods have been devised for causing the color to penetrate through the resistant spore membrane.

In the simplest method a cover-slip after having been prepared in the usual way is covered with Ziehl-Neelsen's (p. 78) carbol-fuchsin solution and held over the Bunsen flame until the fluid steams. This is continued for one or two minutes. It is then washed and dipped in a decolorizing acid solution, such as a 2 per cent. alcoholic solution of nitric acid, or a 1 per cent. solution of sulphuric acid in water, until all visible color has disappeared, then it is washed in running water and dipped for one-half minute in a saturated watery solution of methylene blue. The bodies of the bacilli are blue and the spores red. This same method is also used for staining acid-fast bacilli. Sometimes the spores refuse to take the stain in this manner. We then can adopt *Moeller's method*, which is designed still further to favor the penetration of the coloring matter through the spore membrane. The prepared cover-slip is held for two minutes in chloroform, then washed off in water and placed from one-half to three minutes in a 5 per cent. solution of chromic acid, again washed off in water, and now stained by carbol-fuchsin, which is steamed for several minutes. The staining fluid is then washed off and the preparation decolorized in a 3 per cent. solution o hydrochloric acid or a 5 per cent. solution of sulphuric acid. The preparation is finally stained for a minute in methylene-blue solution. The spores will be red and the body of the cells blue. The different spores vary greatly in the readiness with which they take up the dyes, and we have, therefore, to experiment with each variety as to the length of time it should be exposed to the maceration of the chromic acid. Even under the best conditions it is almost impossible to stain some spores.

*Spore Stain.*—Huntoon has reported the following rapid and reliable method:

4 gm. *acid* fuchsin (Grübler) dissolved in 50 c.c. 2 per cent. aqueous acetic acid.

2 gm. methylene blue (Grübler) dissolved in 50 c.c. 2 per cent. aqueous acetic acid.

Mix the two solutions, shake and set aside for fifteen minutes. Heavy precipitate results. Filter mixture through well-moistened filter paper. Use the filtrate for staining. Reddish-purple filtrate will keep for several weeks. Refilter if precipitate appears.

*To Stain.*—Make a rather thick smear, preferably from an agar slant. Cover smear with dye and steam one minute. Wash in water. Film appears bright red. Dip slide in dilute solution of sodium carbonate (7 or 8 drops saturated solution in tumblerful of water). When film turns blue, rinse immediately in water. Dry and examine. Spores are stained red, the body of the bacillus is stained blue. (Plate III, Fig. 22, shows stained spores.)

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[1] Jour. Am. Med. Assn., 1914, lxii, 18, 1397; also Proc. Soc. Bacteriol., December, 1916.

STAINING ACID-FAST BACTERIA.—The usual stain is that with carbol-fuchsin given under "spore stains." (See Plate VI.)

HERMANN STAIN.—A, crystal violet 3 per cent. solution in alcohol; B, ammonium carbonate 1 per cent. solution in water; mix 1 part of A with 3 parts of B just before using; steam three minutes, decolorize with 10 per cent. nitric acid, wash in alcohol, and counter-stain in Bismarck brown. (See Plate VI.)

**Staining Flagella.**—For the demonstration of flagella, which are possessed by all motile bacteria, we are indebted first to Löffler. The staining of bacterial flagella well is one of the most difficult of bacteriological procedures. In all methods young (twelve-to-eighteen-hour) cultures of agar should be chosen. Enough of the culture to produce slight cloudiness is placed in a few cubic centimeters of filtered tap-water in a test-tube. This may be used immediately, or allowed to stand in the thermostat at blood heat for from one to two hours to permit slight development A tiny drop of this rather thin emulsion is allowed to spread with as little manipulation as possible over the cover-glass so that it may dry quickly. This latter point seems to be the important one, since slow drying allows the bacteria to shed their flagella.

*Bunge's Modification of Löffler's Method* is carried out as follows: Cover-glasses which have been most carefully cleaned are covered by a very thin smear. After drying in the air and passing three times through the flame, the smear is treated with a mordant solution, which is prepared as follows: To 3 parts of saturated watery solution of tannin add 1 part of a 25 per cent. solution of ferric chloride. This mordant should be allowed to stand for several weeks before using. After preparing the cover-slip with all precautions necessary to cleanliness, the filtered mordant is allowed to act cold for five minutes, after which it is warmed and then in one minute washed off. After drying, the smear is stained with the carbol-fuchsin or carbol-gentian violet solution, and then washed, dried and mounted. (Plate III, Figs. 18 to 21.)

Frequently the flagella appear well stained, but often the process has to be repeated a number of times. Overheating of the film prevents the staining of the flagella. The cell membrane may also show by this method.

*Van Ermengen's Method* gives good results. It is as follows: The films are placed for one hour at room temperature, or are heated for five minutes over a water-bath at 100° C. in the following solution:

SOLUTION A.

| | |
|---|---|
| Osmic acid, 2 per cent. solution | 1 part |
| Tannin, 10 to 25 per cent. solution | 2 parts |

Wash successively with water, absolute alcohol and water, then place in the following solution for a few seconds:

SOLUTION B.

0.5 per cent. solution of $AgNO_3$ in distilled water.

Without washing transfer them to a third solution:

SOLUTION C.

| | |
|---|---|
| Gallic acid | 5 grams |
| Tannin | 3 " |
| Fused potassium acetate | 10 " |
| Distilled water | 350 c.c. |

After keeping in this for a few seconds, place again in Solution B until film begins to turn black. Then wash and examine.

**Eosin-methylene-blue Stains.**—These are polychrome staining mixtures introduced by Nocht, Romanowsky and others chiefly for the staining of animal cells, but they are also useful in differentiating bacteria and other germs in tissues and exudates, especially in differentiating those organisms that take ordinary stains faintly, such as the spirochetes. They are fine differential stains for chromatin. Many modifications have been proposed.

In a study of the essential constituents of the Romanowsky stain MacNeal says, both methylene azur and methylene violet are present and participate in the nuclear staining The preparation of solutions directly from the pure dyes, methylene azur, methylene violet, methylene blue and eosin, has been recommended as the best manner of preparing these staining solutions as the proportion of the various constituents may be varied at will to obtain various kinds of differentiation. As a routine blood stain for study of leukocytes and staining of hematozoa, the following is recommended by MacNeal:[1]

| | |
|---|---|
| Solution A. | |
| Methylene azur | 0.3 |
| Methylene violet (Bernthsen's, insoluble in water) | 0.1 |
| Methylene blue | 2.4 |
| Methyl alcohol, pure | 500.0 |
| Solution B. | |
| Eosin, yellowish, water soluble | 2.5 |
| Methyl alcohol, pure | 500.0 |

These solutions keep for at least a year. They are mixed in equal parts and diluted by the addition of 25 c.c. of methyl alcohol to each 100 c.c. of the mixture. This final mixture is employed in the same manner as Leishman's stain. It keeps for a few months.

*Giemsa's Method.*[2]—Smears are fixed in neutralized methyl alcohol for one minute. There are several variations of Giemsa's method. Two of them are given here:

I.

| | |
|---|---|
| Azur II—Eosin | 3.0 gm. |
| Azur II | 0.8 " |
| Glycerin (Merck, chem. pure) | 250.0 c.c. |
| Methyl alcohol (chem. pure) | 250.0 " |

Both glycerin and alcohol are heated to 60° C. The dyes are put into the alcohol and the glycerin is added slowly, stirring. The mixture is allowed to stand at room temperature overnight, and after filtration is ready for use.

The solution is prepared ready for use by Grübler, Leipzig.

One drop of the stain to every cubic centimeter of distilled water, made alkaline by the previous addition of 1 drop of a 1 per cent. solution of potassium carbonate to 10 c.c. of the water, is poured over the slide and allowed to stand for one-half to three hours. The longer time brings out the structure better, and in twenty-four hours well-made smears are not overstained. After the stain is poured off, the smear is washed in running tap-water for one to three minutes, and dried with filter paper. If the smear is thick, the organisms may come out a little more clearly by dipping in 50 per cent. methyl alcohol before washing in water; then the washing need not be so thorough. By this method of staining, the cytoplasm of protozoa stains blue and the nuclear substance a blue red or azur. Young bacteria usually take a dark purple stain, and their metachromatic granules an azur.

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[1] Jour. Infect. Dis., 1906, iii, 412.

[2] Deutsch. med. Wchnschr., 1905, xxxi, 1026.

II.

1.0 gm. azur I (Grübler) in 1000 c.c. distilled water.
0.8 gm. azur II (Grübler) in 1000 c.c. distilled water.
1.0 gm. French eosin in 100 c.c. distilled water.

To mix for use.

9.0 c.c. azur I solution.
9.0 c.c. azur II solution.
0.15 c.c. French eosin solution.
40.0 c.c. distilled water.

Stain one and a half hours at incubatör temperature.

*Wright's Stain.*[1]—One per cent. methylene blue (alcohol rectified) and 0.5 per cent. sodium carbonate are mixed and placed in a steam sterilizer for one hour. When cold, 0.1 per cent. solution of extra B-A eosin (500 c.c. eosin to 600 c.c. methylene-blue solution) is added until the mixture becomes purplish, and a finely granular black precipitate appears. This precipitate is filtered off and dried without being washed. A saturated solution of this is made in pure methyl alcohol. This is filtered and then diluted by adding to 40 c.c. of it 10 c.c. of methyl alcohol. In using, a few drops are placed on the film for a minute; then water is dropped on until a greenish iridescence appears. The stain then remains on for two minutes; then is washed off with distilled water, allowing a little to remain on until differentiation is complete. Dried with filter paper.

*Leishman's Stain.*[2]—This is a modification of *Jenner's stain* which is simply a solution of eosin and methylene blue in methyl alcohol. Instead of ordinary methylene blue, Leishman used the active constituents formed in this stain.

*Solution A.*—To a 1 per cent. solution of medicinally pure methylene blue in distilled water add 0.5 per cent. sodium carbonate and heat at 65° C. for twelve hours, then allow it to stand ten days at room temperature.

*Solution B.*—Eosin extra B-A (Grübler) 0.1 per cent. solution in distilled water.

Mix Solution A and B in equal amounts and allow to stand six to twelve hours, stirring at intervals. Filter and wash the precipitate thoroughly. Collect, dry and powder it. 0.15 gram is dissolved in 100 c.c. of pure methyl alcohol to form the staining solution. It keeps perfectly for at least five months. To stain, cover the dried but unfixed film of blood with the staining solution. After thirty to sixty seconds add about an equal amount of distilled water. Allow this mixture to act for five minutes. Wash in distilled water for about one minute, examining the specimen mounted in water under the microscope. Blot, dry thoroughly, mount in balsam, or preserve the specimen as an unmounted film.

**Methods of Staining Spirochetes.**—Giemsa's method gives excellent results. *Goldhorn's method,* which also gives fine results, is a modification of Giemsa's. It is as follows: Dye; water, 200 cm.; lithium carbonate, 2 grams; methylene blue (Merck's medicinal or a similar preparation), 2 grams. This mixture is heated in a rice boiler with a moderate amount of heat until a rich polychrome has formed. This is determined by examining a sample against artificial light and noting the appearance of a distinctly red color. The solution is allowed to cool and the residue is removed by filtering through cotton. To one-half of this filtrate 5 per cent. acetic acid is gradually added until a strip of litmus paper shows above the line of discoloration a distinct acid reaction. The remaining half of the dye is now added, so as to carry the reaction back to a low degree of alkalinity. A 0.5 per cent. French eosin solution is now added gradually, while the mixture is being stirred until a filtered sample shows a pale bluish color with slight fluorescence. The mixture is allowed to stand for one day and filtered. The precipitate is collected on a double filter paper and dried at a temperature not exceeding 40° C. It is then removed from the filter paper and dissolved in commercial wood alcohol. It is allowed to stand for one day in an open vessel and then filtered.

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[1] Jour. Med. Research, 1902, ii (new series), 138.
[2] British Med. Jour., 1901, p. 635; 1902, p. 757.

To use the stain on smears sufficient dye to cover the smear is dropped on an unfixed preparation and allowed to remain for three or four seconds; the excess is then poured off. The slide is now introduced slowly into clean water with the film side down, is held there for four or five seconds and is then shaken in the water to wash off the excess of dye. It is then allowed to dry and is ready for examination. The pallidum stains violet.

*Silver Impregnation Method.—In Smears.*—Until recently the demonstration in smears of the syphilis spirochete by the *silver impregnation method*, so successfully used by Levaditi in section, has been unsatisfactory. Stern, however, and Flexner corroborating him, have gotten beautiful results by the following simple method:

(*a*) Air-dried in 37° incubator for some hours.

(*b*) Ten per cent. aqueous silver nitrate for some hours (Flexner thinks three or four days' exposure better) in diffuse daylight.

(*c*) When the brownish color reaches a certain tone (easily recognized after experience) and when a metallic sheen develops, the slide is washed well in water, dried and mounted.

The blood cells are well preserved, they have a delicate dark brown contour, and contain fine light brown granules. The spirochetes are deep black on a pale brown and in places a colorless background.

Other spirochetal organisms may be silvered by this method, but as they may be differentiated with greater difficulty than with Giemsa's stain, the latter should always be used as well.

These organisms may also be demonstrated by the India-ink method (see p. 75).

The flagella are brought out by Löffler's method or by the stain recommended by Goldhorn.

*In Sections.*—Sections are prepared by the *silver impregnation method of Levaditi* as follows.[1] Fix small pieces of tissue 0.5 mm. in thickness for twenty-four to forty-eight hours in formalin, 10 per cent. Wash in 95 per cent. alcohol twelve to sixteen hours. Wash in distilled water until the pieces sink. Impregnate two or three hours at room temperature and four to six hours at 50° C. in the following fluid: Nitrate of silver, 1; pyridin, 10 (add just before using); distilled water, 100. Wash rapidly in 10 per cent. pyridin. Reduce the silver by placing in the following mixture for several hours: Pyrogallic acid, 4; acetone, 10 (add just before using); pyridin, 15; distilled water, 100. Harden in alcohol, xylol, paraffin. Levaditi's first method is longer but more reliable. Fix small pieces in formalin, 10 per cent. Harden in 95 per cent. alcohol. Wash in distilled water several minutes. Impregnate three to five days at 37° C. in 1.5 per cent. solution silver nitrate. Reduce twenty-four hours in: Pyrogallic acid, 4; formalin, 5; water, 100. Imbed in paraffin. By these methods the spirochetes appear densely black.

**Ross's Method of Examining a Large Quantity of Malarial Blood in One Film.**—A large drop of blood (about 20 c.mm.) is placed on a glass slide and is slightly spread over an area which can be covered by an ordinary cover-glass. This is allowed to dry in the air or it is warmed over a flame without heating it more than enough to fix the hemoglobin. The dry film is then covered with an aqueous solution of eosin (10 per cent.) and allowed to remain about fifteen minutes. This is then gently washed off and a weak alkaline methylene-blue solution is run over the film and left for a few seconds, when the preparation is again gently washed. After drying, it is ready for examination.

**The Eosin-methylene-blue Method Recommended by Mallory for Tissues may be Used for Smears as Follows.**—The smears are fixed in Zenker's solution for one-half hour; after being rinsed in tap-water they are placed successively in 95 per cent. alcohol + iodin, one-quarter hour; 95 per cent. alcohol, one-half hour; absolute alcohol, one-half hour; eosin solution, twenty minutes, rinsed in tap-water; methylene-blue solution, fifteen minutes, differentiated in 95 per cent. alcohol from one to five minutes; and dried with filter paper.

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[1] Levaditi and Manouelian: Compt. rend. Soc. de biol., 1906, lx, 134.

**Staining Method for Negri Bodies** (*Williams's Modification of Van Giesen's Method*[1]).—Smears partially air-dried are fixed for ten seconds in neutral methyl alcohol to which 0.1 per cent. picric acid has been added. Excess of fixative removed by filter paper. Smears then stained in following solution: Saturated alcoholic solution fuchsin, 0.5 c.c.; saturated alcoholic solution methylene blue, 10 c.c.; distilled water, 30 c.c. The stain is poured on the smear and held over the flame until it steams. The smear is then washed in tap-water and blotted with fine filter paper. The Negri bodies are magenta, the nerve cells blue, and the red blood cells yellow, or salmon color (Plate X, Fig. 1). This staining mixture may be kept in the ice-box for a long time.

**Heidenhain's Iron-hematoxylin Stain.**—(*a*) Mordant and differentiating fluid: Iron oxyammonium sulphate, 2.5 grams; distilled water, 100 c.c. (*b*) Staining fluid: Hematoxylin, 1 gram; alcohol, 10 c.c.; distilled water, 90 c.c. (To be kept in a red bottle and allowed to stand for about four weeks before using.) For use see chapter on Amebæ.

**Preservation of Smears.**—Dry stained preparations of bacteria keep indefinitely, but if mounted in Canada balsam, cedar oil, or dammar lac they tend gradually to fade, although many preparations may be preserved for many months or years. Dry unstained spreads should be kept in the ice-box until stained.

**Examination of Microörganisms in Tissues.**—Occasionally it is of importance to examine the organisms as they occur in the tissues themselves. The tissues should be obtained soon after death, so as to prevent as much as possible postmortem changes, with consequent increase or decrease in the number of microbes. Selected pieces of tissues can be frozen by ether or carbon dioxide and sections cut, but the best results are obtained when the material is imbedded in paraffin or in celloidin.

**Fixing and Hardening Tissues.**—From properly selected portions small pieces, not larger than ¼ inch by ⅛ inch, are removed and placed in one of the following fixatives:

1. *Absolute Alcohol.*—Absolute alcohol for from four to eight hours, and longer if thicker. For the larger pieces it is better to change the alcohol after eight hours. The pieces of tissue should be kept from falling to the bottom, as the higher layers of alcohol remain nearer absolute. If along with the microorganisms one wishes to study the finer structure of the tissue, it is better to employ one of the other fixatives.

2. *Formalin.*—For fixing in formalin the tissue is put in 4 to 10 per cent. formalin solution (the 40 per cent. commercial solution) for three to twenty-four hours, and then in successive strengths of alcohol.

3. *Corrosive Sublimate.*—Corrosive sublimate (saturated solution in 0.75 per cent. sodium chloride solution) is an excellent fixing agent. Dissolve the sublimate in the salt solution by heat, allow it to cool; the separation of crystals will show that saturation is complete. For pieces of tissue ⅛ inch in thickness four hours' immersion is sufficient; for larger, twenty-four hours may be necessary. They should then be placed in pieces of gauze and left in running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate.

4. *Sublimate Alcohol.*—Hot sublimate (saturated) alcohol (50° C.) or saturated sublimate, to which 5 per cent. glacial acetic acid may be added. The

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[1] Am. Jour. Pub. Hyg., 1908, xviii, No. 1.

preparation should remain in it a few seconds, then should be washed for one-half hour in 60 per cent. iodin-alcohol, and then placed in 70 per cent. alcohol. They may remain here for an indefinite time, until they are to be stained, when they are rinsed in distilled water and then placed in the staining fluid.

5. *Osmic Acid.*—Two per cent. osmic acid (to be kept in a red glass with a ground-glass stopper). Moist smears are exposed to its fumes for a few seconds, small pieces for sections, four to eight hours, then carried through the various alcohols and xylol and mounted or imbedded in the usual way.

6. *Hermann's Fluid.*—A 1 per cent. solution platinum chloride, 15 c.c.; a 2 per cent. solution osmic acid, 4 c.c.; glacial acetic acid, 1 c.c. Moist spreads may be fixed for several minutes; very small pieces of tissue for twenty-four hours.

7. *Zenker's Fluid.*—Add to a solution of Müller (potassium bichromate, 2 to 2½ parts; sodium sulphate, 1 part; water, 100 parts) 5 per cent. of saturated sublimate solution and, when ready to use, 5 per cent. of glacial acetic acid. Moist spreads are fixed for one to five minutes, small pieces of tissue for three to twelve hours. They are then washed with water or put immediately into successive alcohols.

To *harden* they are placed successively for twenty-four hours each in the following strengths of ethyl alcohol: 30 per cent., 69 per cent. and 90 per cent. Finally they are placed in absolute alcohol for twenty-four hours which dehydrates them and they are then ready to be imbedded in paraffin.

*To imbed in paraffin*, the pieces are put in: (1) Cedar oil until translucent; (2) cedar oil and paraffin, equal parts, at 37° C. for two hours; (3) paraffin 52° C. two hours in each of two baths. They are then boxed ready for sections. Sections are cut at 3 to $6\mu$, and are dried at 36° C. for about twenty-four hours, protected from dust. Xylol may be used instead of cedar oil.

The paraffin sections of tissue having been prepared and cut, they are ready for staining after the paraffin is removed. If all of the sublimate has not been removed by the water, the sections may be immersed in iodin-alcohol for ten minutes.

Löffler's Staining Method for Sections.—The section is placed in Löffler's alkaline methylene-blue solution for five to thirty minutes, decolorized for a few seconds in 1 per cent. acetic acid. It is then placed in absolute alcohol, xylol and Canada balsam. The number of seconds during which the preparation remains in the acetic acid must be tested by trials.

Stain for Influenza Bacilli in Tissues.—MacCallum[1] recommends a combination of Goodpasture's and Weigert's stains as follows:

Fixation should be in Zenker formaldehyde solution, and very thin paraffin sections should be used.

1. Stain for from ten minutes to one-half hour or more in Goodpasture's stain, which is:

| | Gm. or c.c. |
|---|---|
| 30 per cent. alcohol | 100.00 |
| Basic fuchsin | 0.59 |
| Anilin | 1.00 |
| Phenol crystals | 1.00 |

2. Wash in water.
3. Differentiate in 40 per cent. formaldehyde solution. This requires only a few seconds. The bright red color washes away and gives place to a clear rose.
4. Wash.
5. Counterstain in saturated aqueous picric acid. The section remains until it assumes a purplish yellow, about three to five minutes or less.
6. Wash in water.
7. Differentiate in 95 per cent. alcohol. The red reappears and some of it is washed out. Some of the yellow of the picric acid is also washed out.

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[1] Jour. Am. Med. Assn., 1919, lxxii, 193.

8. Wash in water.
9. Stain in Stirling's gentian violet five minutes or more.
10. Wash in water.
11. Gram's iodin solution.
12. Blot dry without washing.
13. Anilin and xylene (equal parts) until no more color comes away.
14. Two changes of xylene.
15. Balsam.

Gram-negative organisms stain red, gram-positive blue. The tissues stain brilliantly in shades of red and purple. Fibrin is, of course, sharply stained, and the granules of mast cells are prominent as red or purplish coccus-like bodies.

The method should be useful in the study of infections of exposed surfaces, such as the intestinal mucosa and the urinary bladder, with gram-negative organisms. The lesions of dysentery, typhoid fever and many other similar conditions might be studied with advantage by this method.

**Methods of Examination of Ringworm Fungus.**—*Living Specimens.*—Place scales or hairs in warm liq. potassæ (20 per cent.) for a few seconds. Examine under cover-glass.

*Permanent Specimens.*—Remove fat with chloroform, then place material in formic acid and heat to boiling (two or three minutes). Remove acid by washing in distilled water, stain with Löffler's methylene blue. Wash, dehydrate in absolute alcohol, clear in xylol and mount in balsam.

Sabouraud's media are best for growth of the organism (p. 110). Upon this medium within half a dozen days septate mycelial threads with chlamydospores (p. 27) are seen. Reddish or brown pigment sometimes develops.

# CHAPTER IV.

# GENERAL METHODS USED IN THE CULTIVATION OF MICROÖRGANISMS.

## GLASSWARE, CULTURE MEDIA, STERILIZATION, METHODS OF CULTIVATION AND ISOLATION, CULTURAL CHARACTERISTICS.

THE methods employed for the artificial cultivation of microörganisms are of fundamental importance. By their use we can obtain one variety growing apart from all others, namely, in pure culture. This pure culture may be planted on various media and the morphological, biochemical, and cultural characteristics studied for classification and identification. It is evident that all glassware and instruments used must be free from other microörganisms; that is, they must be sterile.

## GLASSWARE.

Various types of glassware are needed such as test tubes, flasks, bottles, petri dishes (often called "plates"), pipettes, etc. (Figs. 14 to 20). Before glassware is subjected to the necessary sterilization, noted above, it should be chemically clean.

### Preparation of Glassware.

New glassware requires not only a thorough mechanical cleansing with soap and hot water and the loosening of adherent dirt by means of test-tube or bottle brushes, but also special attention to render it free from the *resistant spores* which are often present in the straw or other packing material. Such spores resist frequently the high temperatures of hot-air sterilization (p. 91), but they may be destroyed by boiling the glassware in a strong soap solution (5 per cent.) for one hour. Since many of the soap powders cloud glassware they are to be avoided; instead add to the water thin shavings of a good brown soap.

Old glassware containing cultures, especially those of tetanus and anthrax, should be sterilized in the autoclave at 15 pounds' pressure for one-half hour; less resistant cultures may be killed by boiling for one hour in a covered boiler, containing a 5 per cent. soap solution which should cover and fill the glassware from which all stoppers have been removed. The cotton stoppers should be disinfected by burning or other means. Any solid medium present is melted and the glassware then washed according to the directions given above. The *rinsing* must be thoroughly done in running water to remove the soap. The glassware should then be allowed to drain and dry before being plugged.

**Neutralization of Glassware.**—Where slight changes of reaction are important the glassware should be neutralized after washing. Soak in 1 per cent. hydrochloric acid for several hours or boil for one-half hour, wash free of acid, rinse in tap water then in distilled water. New glassware, especially of the cheaper grades is most likely to give off free alkali.

**Plugging.**—All narrow-mouthed containers are plugged in such a way as to exclude dust and germs. Use ordinary non-absorbent cotton (cotton batting).

The cotton should not be twisted in, as creases will form along the glass, leaving channels for contamination. Either fold into a plug or take a square of the cotton, fold one corner, place a rod against the fold to push the cotton into the neck of the container or tube. A sufficient amount of cotton should project for handling and the plug should be just tight enough to allow one to lift the container by means of the plug. Several thicknesses of filter paper may be used to cover beakers and other wide-mouthed containers.

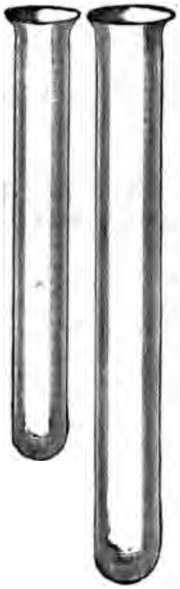


FIG. 14.—Test-tubes for small amounts of media and individual cultures; if agar, either slanted or in 10-c.c. amounts to be melted and poured in petri dishes. They can be also used without lip. Average size, 6 x ⅝ inches.

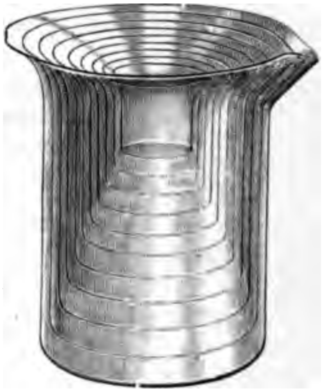
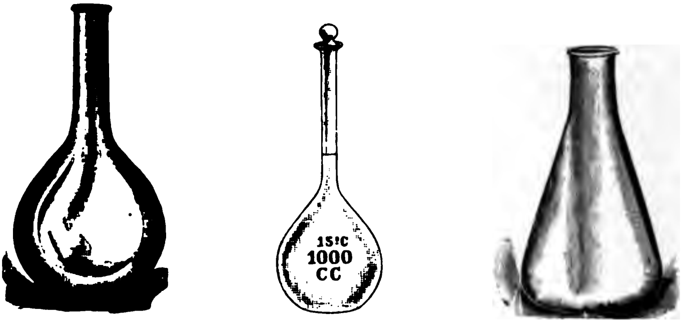


FIG. 15.—Beakers.



Globe. Volumetric. Erlenmeyer.

FIG. 16.—Flasks for preparing culture media (collecting filtrates) or for growing bacteria in liquid media, as for production of diphtheria and tetanus toxin. Capacity 500 to 2000 c.c.

**Sterilization.**—After the glassware is plugged it is sterilized by dry heat. Any type of hot-air sterilizer may be used (Fig. 21). Heat at a temperature of 160° to 170° C. for one hour. This heating not only sterilizes but sets the shape of the cotton plugs. Keep the sterilizer closed when the sterilization is finished so that the cooling may be gradual, otherwise the glassware may crack. When large amounts of glassware must be handled the use of a good thermo-regulating valve on the sterilizer together with a recording thermometer, gives excellent results and releases the attendant for other work.

Fig. 17.—Type of bottle commonly used for dilutions, etc., or as substitutes for flasks, capacity 100 c.c.

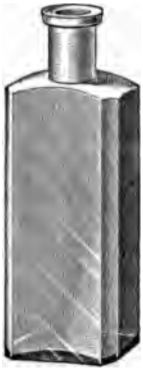


Fig. 18.—Blake bottle. Laid on its flat side it gives a large surface of broth or agar. For preparation of vaccines and tuberculin, capacity, 1 quart and 1 pint.

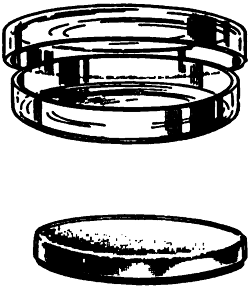
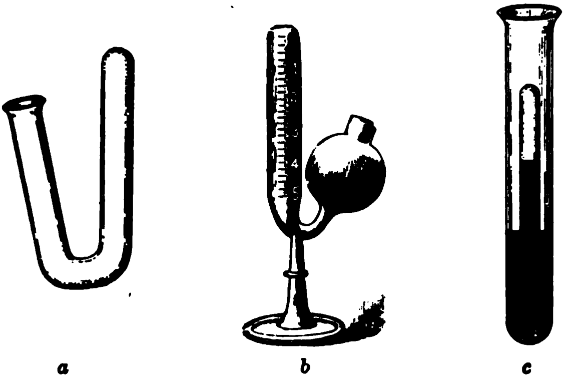


Fig. 19.—Petri dishes. Commonest size is 10 c.c. in diameter. For solid media only, chiefly for mixed cultures; either surface growth or scattered through medium (agar or gelatin) while in liquid state: medium then hardens in plate. Individual colonies, growing separately after incubation, can be fished and transferred to separate tubes.



*a* *b* *c*

Fig. 20.—Types of fermentation tubes for liquid media to show production of gas by inoculated organisms. The gas collects in closed portion of tube displacing liquid.

## CULTURE MEDIA.

### General Considerations.

Most microörganisms causing disease require complex foodstuffs of suitable reaction (see Reaction) and similar in constitution to those in the animal body. The general basis of media for these types is an *infusion* or *watery extract of meat* which contains soluble albumins, extractives, salts, carbohydrates and coloring matter. In many respects this infusion of meat would make a good medium, but the application of heat necessary for its sterilization affects some of its constituents deleteriously by changing their composition, thus causing their loss as foodstuffs. After such changes a very clear medium may be obtained by filtration but the lost nutritive material must be replaced in part at least by the addition of some other substance. *Peptone*, which is soluble in water and is not precipitated by boiling, fills this need to quite an extent.

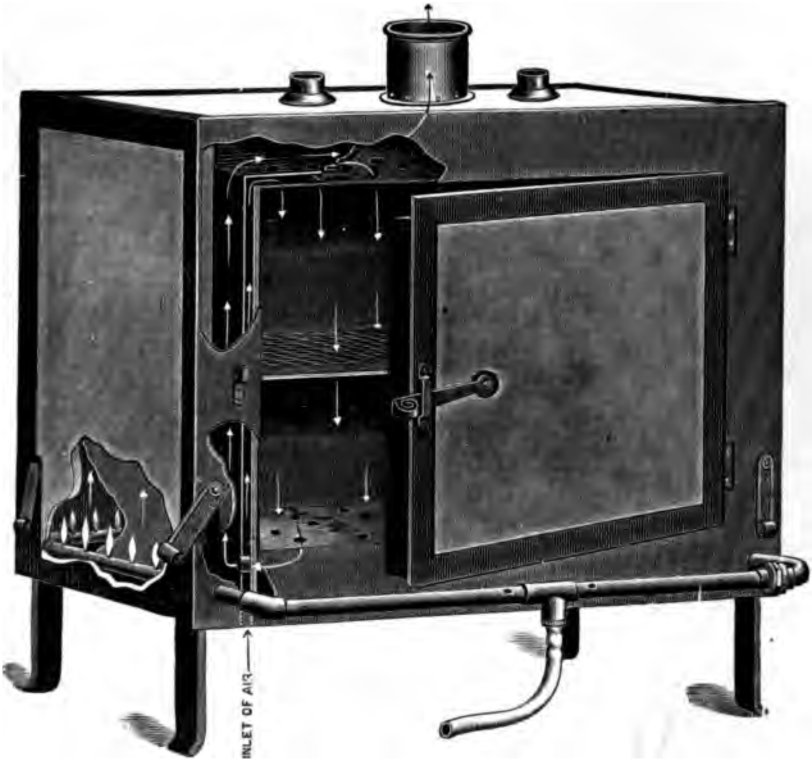


FIG. 21.—Hot-air sterilizer. Lautenschläger form.

As an alternative basis for the above infusion of meat, potted *beef extract* (Liebig's, Armour's, etc.) may be used. It contains extractives or meat flavor and mineral salts but the prolonged heating in the method of manufacture causes a great loss of its nutritive elements. The addition of peptone restores these in part only. Beef extract is, however, very useful for certain kinds of media and is of great convenience in small laboratories or those in tropical regions.

To meet the needs of some of the pathogenic organisms other nutritive substances, such as serum or blood, are frequently added to the sterilized medium. Carbohydrates may also be added. On the other hand, the non-pathogenic organisms vary in their ability to flourish on these more complex media, for some will grow only in simple media containing inorganic salts.

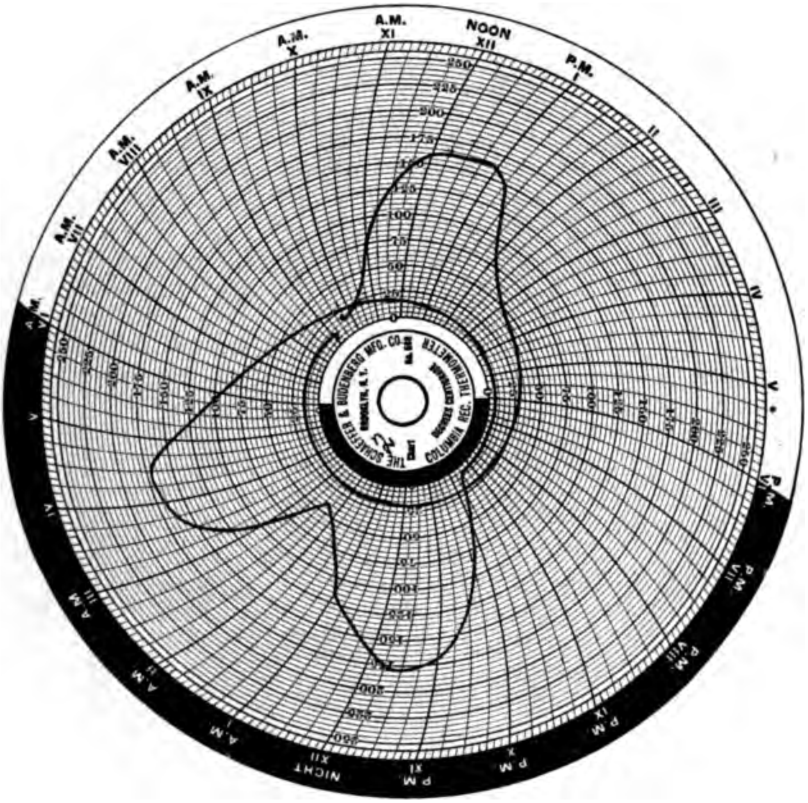


FIG. 22.—Recording chart of hot-air sterilizer.

The media outlined above are fluid in character but their consistency may be modified by the addition of suitable amounts of gelatin, agar or albumin. The first two solidify when cooled and the third is coagulated by heating; thus they become either *solid* or *semisolid* as desired. *Gelatin* gives a very clear transparent solid medium but as its melting point (20° C.) is below that required for the growth of pathogenic organisms (37° C.) its usefulness is limited. *Agar*[1] a substance of vege-

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[1] The ordinary market agar is a variable product. Although it is "graded" commercially there is no way of telling whether or not a grade is suitable for bacteriological purposes unless it is tested. The solidifying quality depends, as with fruit jellies, on the pectin content. It is advisable, therefore, to test a sample of each lot ordered for this substance. The pectin should be present to the extent of at least 90 per cent.

*Test for Pectin.*—Wash 8 to 10 grams of thread agar rather rapidly in cold water. Dry at 37° C. overnight. Weight out 5 grams and dissolve them in 1000 c.c. of distilled table origin, melts just below the boiling-point (100° C.) and remains liquid until cooled down to about 40° C. It is quite clear if properly made. *Coagulated albumin*, as typified in Löffler's blood serum, provides both firmness and a certain amount of food, but has the drawback of being opaque.

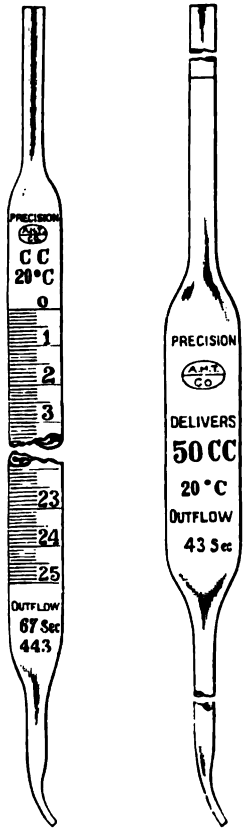

FIG. 23

Graduated pipette. Volumetric pipette.

Obtainable in various sizes and graduations for measuring accurately definite amounts of fluids in preparation of dilutions, etc.

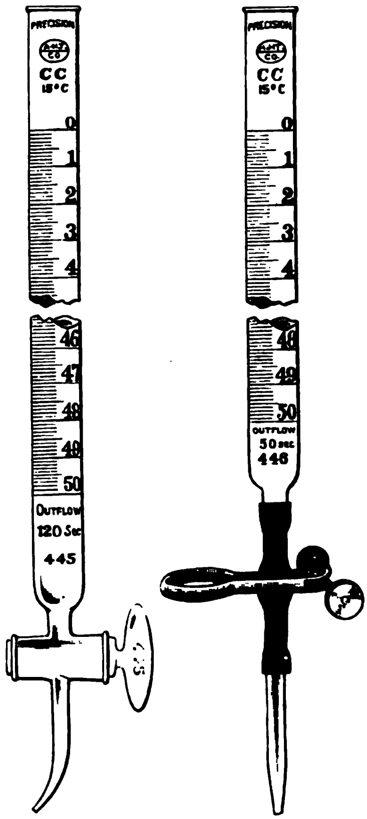

FIG. 24

Burettes. Most convenient type has a blue line against a white background on back, giving sharper readings; to be held in clamp on ring-stand; used in titration.

*Solid media* were first designed for keeping colonies of organisms separate so they could be fished and pure cultures obtained. Such

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water to which has been added 2 per cent. of sodium chloride. The dissolving of the agar may be done by boiling on the stove or by heating in the autoclave or Arnold. Add 2 liters of alcohol (95 per cent.) to precipitate the pectin. Let stand overnight. Dry a filter paper overnight at 37° C. and then weigh it. Filter the mixture through the weighed paper. Dry the residue on the paper at 37° C. Weigh again, calculate the amount of residue and then calculate the percentage.

media have been found excellent for other purposes generally and are more used than fluid media.

*Semisolid media,* prepared either with agar in lessened amounts or agar and gelatin mixed, are especially useful for keeping routine stock cultures alive for long periods of time without transplanting.

## Method of Preparing Culture Media.

The meat used most generally is either beef or veal. It must be carefully freed from fat, fascia, etc. This is done preferably at the laboratory just before the meat is chopped fine or put through the meat grinder. Chopped meat bought from the butcher, as such, is not desirable, as it contains too much fat. In ordering meat allowance should be made for the waste of fat and bone. Beef hearts, if obtained fresh, are economical and have been found satisfactory for various purposes.

### Nutrient Broth.

Chopped meat (veal or beef), 5 pounds.[1]
Tap water, 5000 c.c.
Soak overnight at ice-box or at room temperature.

↙ ↘

**Method I.**

Strain through cheese-cloth; squeeze by twisting cloth or use meat press. Discard raw meat. Fluid = *meat juice.*

**Method II.**

Weigh kettle and contents. Heat at 45° C. for one hour, then boil half hour. Make up by weight loss due to evaporation by adding hot water. Strain through cheese-cloth. Squeeze by twisting cloth or use meat-press. Discard cooked meat. Fluid = *meat infusion,* which may be sterilized in flasks and stored for future use.

↘ ↙

Add: Peptone, 1 per cent.
Sodium chloride, 0.5 per cent.

↙ ↘

Heat to 50° C. to dissolve peptone and salt. Determine reaction (see Reaction). If phenolphthalein is used as indicator, boil in casserole to approximate roughly future conditions. ↘

Heat to boiling. Determine reaction (see Reaction). With phenolphthalein as indicator, use room temperature method of titration. ↙

Set reaction by adding calculated amount of sodium hydroxide (normal). Boil or autoclave for ten minutes, to throw down precipitate.

Determine reaction again, using room temperature method. Adjust reaction if necessary. (In this case boil again). Filter through cotton and filter paper in glass funnel. Sterilize in tubes or flasks by heating in autoclave for half hour at 15 pounds' pressure.

*Explanation of Methods.*—In Method I less heat is used with consequently less change of nutritive material. The whole process of making the broth is carried out at one time. It is however somewhat wasteful in that the raw meat still containing some nutriment is discarded. On the other hand the broth is particularly suitable for some special purposes—such as the preparation of pneumococcus vaccine or the growth of other delicate organisms.

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[1] These directions are for an amount larger than the 1 liter often suggested, because the proportionate loss by evaporation is much less.

With Method II the greater amount of heating causes greater loss of foodstuffs especially if the infusion is sterilized for storing with the reaction (see Reaction) unadjusted. The advantages however of having on hand stock meat infusion ready for further preparation in various ways, outweigh the objections, since media made from such stock give excellent results in general work.

The following *variant* is sometimes useful in place of nutrient broth:

**Beef Extract Broth.**—Beef extract (Liebig's or Armour's, etc.), 2 to 5 grams; water, 1000 c.c.; peptone (1 per cent.), 10 grams; sodium chloride (0.5 per cent.), 5 grams.

Heat to boiling over fire to dissolve ingredients. Boil fifteen minutes. Determine reaction (see Reaction) and adjust if necessary by addition of normal sodium hydroxide. Boil ten minutes to throw down precipitate. Filter through cotton and paper in glass funnel. Put in tubes or flasks and sterilize in autoclave one-half hour at fifteen pounds' pressure.

**Reaction.** — General considerations. The meat bases for culture media and also peptones are naturally more or less acid in comparison with true neutrality or distilled water (page 99). Most pathogenic organisms grow best in media with a reaction near true neutrality or slightly alkaline to it, therefore it is necessary to adjust the reaction of media to the most suitable point by the addition of an alkali such as sodium hydroxide or sodium carbonate.

The oldest methods were very simple—litmus or litmus paper (p. 101) which changes color at about true neutrality was used as an indicator (p. 100) and the acidity reduced to neutral by adding a 4 per cent. solution of sodium hydroxide. Later phenolphthalein was introduced together with the titrimetric method. Its errors are pointed out on page 103. The methods recently introduced for obtaining the hydrogen ion concentration or actual acidity (p. 97) of culture media give much more accurate results.

**Determination of Reaction.**[1]—The determination of the true reaction, *i. e.*, actual acidity, requires an understanding of the theory of hydrogen ions. This theory of electrolytic dissociation was brought forward in 1887 by Arrhenius. When a substance of a certain class (acid, salt or base) goes into solution in water some of it remains in the form of molecules while other molecules are split in two parts—positive ions carrying a positive charge and negative ions carrying a negative charge of electricity. *Example.*—Normal hydrochloric acid (HCl), which is a solution of the acid in water, contains molecules of HCl to the extent of 22 per cent., while the dissociated portion is made up of positive hydrogen ions (H-ions) and negative chlorine ions (Cl-ions) which together form 78 per cent. of the whole.

Substances in the state of ions are entirely different from molecular substance; for example, dry hydrogen (molecular) is tasteless and not acid. On the other hand, hydrogen *ions* are acid and give a watery solution containing them its sour taste and other acid properties. If their electrical charge is removed by withdrawing the solvent water, two H-ions unite to become a molecule of hydrogen (H) and lose their acid properties.

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[1] This explanation is based chiefly on two articles (1) A. L. Wachter: Personal Communication. (2) H. J. Conn, Chairman: Methods of Pure Culture Study. Report of Committee on the Chart for Identification of Bacterial Species, Jour. Bacteriol., March, 1919, iv, 2, 107.

In an aqueous solution of sugar the molecules are not dissociated to any extent, if at all. Solutions of this character do not conduct electricity.

Pure water ($H_2O$) contains both positive ions (H) and negative ions (OH) in equal amounts which neutralize each other. $\overset{\curvearrowleft}{\underset{\curvearrowright}{\underset{+}{H} + \underset{-}{OH}}}$ If to pure water we add a drop of HCl we have a mixture containing H-ions, OH-ions and Cl-ions. The number of free H-ions is greatly increased because of the large number in the drop of HCl. The number of OH-ions is decreased because the change in ionic equilibrium caused many to combine with free H-ions to form water. If a larger amount of HCl is added the H-ion concentration increases and the OH-ion concentration decreases. If a *base* is added to water or other neutral solution the relation is exactly the reverse—the concentration of OH-ions is increased and the concentration of H-ions decreased. Either the H-ion concentration or the OH-ion concentration may be used as an *index* (p. 99) but it is customary to use the H-ion concentration which is inversely proportional to the dissociation of the base and therefore to the true alkaline reaction of the solution.

The degree of dissociation or ionization of different substances in solution in water varies. In dilute solutions nearly all of the molecules are dissociated; in stronger solutions the molecules are less dissociated. Acids and bases dissociate or ionize to a greater extent than water and in turn the strong inorganic acids, as sulphuric and hydrochloric, ionize to a greater extent than the weaker acids such as acetic and other organic acids. The H-ion content or concentration of a solution increases with dissociation or ionization, therefore, if different acids are dissolved in the same ratio to water, the H-ion concentration is an index of the strength of each acid. Examples—normal (N/1) acetic and decinormal (N/10) hydrochloric acids in chart 104).

*Normal Acid.*—The older definition reads "one containing in one liter as much acid as contains 1 gram-atom of replaceable hydrogen." The newer definition is as follows: "A solution containing sufficient acid to furnish 1 gram of ionizable (*i. e.*, capable of dissociation) hydrogen per liter." All the hydrogen, however, may not be dissociated at one time. For example, we have seen (p. 96) that in a normal solution of HCl only about 78 per cent. (or 0.78 of a gram) is dissociated or in the form of ions at 20° C. The remaining 22 per cent. (0.22 gram) is combined with chlorine as molecules of HCl. In *decinormal* HCl, at 20° C., about 92 per cent. of the hydrogen is dissociated or in the form of ions. In higher dilutions the dissociation or ionization is almost complete.

*Normal Alkali.*—Just as a liter of N/1 acid contains 1 gram of ionizable hydrogen so a liter of N/1 alkali contains an amount of ionizable hydroxyl chemically equivalent to the 1 gram of hydrogen; for example, normal sodium hydroxide (NaOH) contains 17 grams of OH (atomic weight of $O = 16$, of $H = 1$).

The amount of dissociation occurring in any solution is affected by the degree of dilution, temperature, presence of other substances known as *buffers*, and also other factors. A *buffer* is any substance (present in a solution) which because of its affinity for either acid or alkali tends to preserve the original H-ion concentration of the solution by combining with any acid or alkali which may be added thus nullifying their effect. Peptone, meat infusion, sodium borate, dipotassium phosphate, etc., act as buffers.

The *actual acidity* of a medium depends upon the amount of ionized hydrogen present, *i. e.*, on the H-ion concentration under the existing conditions of temperature, buffers, etc., in other words on the amount of dissociated acid (or alkali) present at a given time. The *total acidity* takes into account all the acid (or alkali) present whether dissociated or undissociated. For example, we have seen that a liter of N/1 HCl contains 1 gram of ionizable hydrogen but that only 78 per cent. of this hydrogen is actually ionized. If now a little N/1 alkali is added, some of the dissociated acid is neutralized and at once some of the previously undissociated acid becomes ionized or dissociated to take the place of that portion neutralized. Further additions of the alkali would continue the process until no undissociated acid remained and the H-ions of the dissociated acid would be neutralized.

The usefulness of a medium depends upon its actual acidity and whether this is suitable for the particular organisms which are to be cultivated. This actual acidity, or H-ion concentration, may be determined in either of two ways: (1) electrometrically, (2) colorimetrically. The first is an accurate method and one used chiefly in careful determinations in physical chemistry. The second is based on the first and while it is not quite so accurate the error is but slight. Its comparatively easy application makes it generally useful especially for bacteriological work.

**Electrometric Method.**—The medium to be tested is made to act the part of the solution in an improvised battery. By means of special electrodes the slight current, derived from the electrically charged ions in the medium, is carried to and combined with the known current of a standard cell. With the aid of a potentiometer the combined current is read, the increase above the known current noted and, by means of mathematical formulæ, the H-ion concentration is calculated. This method is too complicated and difficult for ordinary purposes.

**Colorimetric Method.**—It has been found that certain shades of color are obtained when certain indicators (p. 100) are added each to a specially prepared solution of known H-ion concentration as determined by the electrometric test. Such solutions are known as standard solutions (see below). If a suitable one of the indicators be added to a medium or a solution, whose H-ion concentration we wish to determine, a similar shade of color appears. By deciding which shade of color in the standard set of solutions the shade of the medium (plus the indicator) approaches most nearly we can determine quite closely its H-ion concentration.

The color of the culture medium and in some instances its turbidity are the chief sources of error.[1] This changing or masking of the indicator color may be obviated in two ways: (1) It must be remembered that all successful media are buffer solutions; that is, they contain salts that require relatively large changes in acid or base to produce small changes in reaction. The hydrogen ion concentration of these solutions is not changed materially by the addition of water. It is possible therefore to dilute the color of the medium itself without affecting the reaction. (2) The color of the medium may be superimposed upon that of the indicator by the comparator method introduced by Walpole. (See Comparator Block under Use of Newer Indicators.)

**Standard Solutions.**—The standard solutions used in the colorimetric method of determining H-ion concentrations are buffer solutions consisting generally of mixtures of some acid and its alkali salt. (Clark and Lubs). These mixtures have definite H-ion concentrations as determined by the electrometric test. They may be prepared either as suggested by Clark and Lubs[2] or after the method of Sörensen. While the range of the mixtures (Sörensen) given below is somewhat limited it will be found useful for many culture media.

*Sörensen's Standard Phosphate Solutions.*—Make a M/15 solution of each salt using ammonia-free distilled water:

[3]$Na_2HPO_4$ (anhydrous) 9.47 grams per liter.

$KH_2PO_4$, 9.08 grams per liter.

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[1] O. T. Avery: Personal Communication.

[2] Jour. of Bacteriology, 1917, ii, 1.

[3] Ordinary "sodium phosphate" containing an unknown amount of water of crystallisation must not be used. This salt and also the $KH_2PO_4$ especially prepared for this purpose can be obtained from a reliable drug house. Ask for Sörensen's **potassium phosphate** and sodium phosphate.

Prepare mixtures[1] as follows using the greatest accuracy in pipetting:

| M/15 $Na_2HPO_4$. c.c. | | M/15 $KH_2PO_4$. c.c. | | H-ion concentration expressed in $P_h$ value.[2] |
|---|---|---|---|---|
| 97.5 | + | 2.5 | = | $P_h$ 8.3 |
| 95.0 | + | 5.0 | = | $P_h$ 8.0 |
| 92.0 | + | 8.0 | = | $P_h$ 7.8 |
| 88.0 | + | 12.0 | = | $P_h$ 7.6 |
| 82.0 | + | 18.0 | = | $P_h$ 7.4 |
| 73.0 | + | 27.0 | = | $P_h$ 7.2 |
| 62.0 | + | 38.0 | = | $P_h$ 7.0 |
| 50.0 | + | 50.0 | = | $P_h$ 6.8 |
| 37.0 | + | 63.0 | = | $P_h$ 6.6 |
| 26.0 | + | 74.0 | = | $P_h$ 6.4 |
| 18.0 | + | 82.0 | = | $P_h$ 6.2 |
| 12.0 | + | 88.0 | = | $P_h$ 6.0 |
| 8.0 | + | 92.0 | = | $P_h$ 5.8 |

A set of tubes which is more easily prepared and which gives roughly the same shades of color (when an indicator is added), as the Standard set, is suggested by Barnett and Chapman.[3]

Select 12 test-tubes of even caliber and place in two rows of 6 each. In each tube of one row put 5 c.c.[4] of a dilute alkali (twentieth normal sodium hydroxide). In each tube of the other row put 5 c.c. of very dilute acid (1 drop of concentrated $H_2SO_4$ or HCl to 100 c.c. of distilled water is sufficient. Avoid stronger acid).

Add the indicator (either brom thymol blue or phenol red) as follows:

| Acid tubes. | Alkali tubes. | | H-ion concentration. Brom thymol blue. | Phenol red. |
|---|---|---|---|---|
| 9 drops | 1 drop | = | $P_h$ 6.2 | $P_h$ 6.9 |
| 8 " | 2 drops | = | $P_h$ 6.4 | $P_h$ 7.2 |
| 7 " | 3 " | = | $P_h$ 6.7 | $P_h$ 7.5 |
| 6 " | 4 " | = | $P_h$ 6.9 | $P_h$ 7.7 |
| 5 " | 5 " | = | $P_h$ 7.1 | $P_h$ 7.9 |
| 4 " | 6 " | = | $P_h$ 7.3 | $P_h$ 8.1 |

The tubes are to be viewed in pairs of acid and alkali, each pair containing the sum of ten drops of indicator.

The figures ($P_h$ value) which are used to represent the H-ion concentration are obtained as follows: Distilled water ($H_2O = H + OH$) represents true neutrality for the positive hydrogen (H) ions and negative hydroxyl (OH) ions are present in balanced amounts. As fast as dissociation occurs the H-ions and OH-ions recombine. One liter of distilled water contains $\frac{1}{10,000,000}$ gram of ionizable hydrogen. This awkward fraction may be written 0.0000001 or $1 \times 10^{-7}$ = log.$^{-7}$ (minus exponent because of the decimal). The "log" is dropped, together with the minus sign, and for convenience the term $P_h$, as suggested by Sörensen, substituted. Therefore, the amount of ionizable hydrogen present in distilled water is represented by $P_h$ 7. (See p. 100.) The other values are obtained in a like manner. Since $P_h$ is an invert logarithm or the reciprocal of H-ion concentration it decreases with an increasing H-ion concentration. Therefore, acid solutions have a $P_h$ value less than 7, and basic solutions a $P_h$ value greater than 7; or, a solution containing an amount of H-ions greater than $\frac{1}{10,000,000}$ gram per liter is acid; one containing less than $\frac{1}{10,000,000}$ gram per liter is alkaline.

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[1] These mixtures retain their values for three or four weeks. For their proper use, see Newer Indicators.

[2] $P_h$ as used by Sörensen represents the exponent of the logarithm (to the base 10) of the hydrogen ion concentration.

[3] Jour. Am. Med. Assn., 1918, lxx, 1062.

[4] If desired, 10 c.c. may be put in each tube and the indicator solution measured in tenths of a cubic centimeter instead of in drops.

HYDROGEN ION CONCENTRATION.[1]

|  | $P_h$ values.[2] | Acid and alkali normalities. | $C_h$ values[3] or grams per liter of hydrogen ions. |
|---|---|---|---|
| Acid: | $P_h$ 0.0 | $\frac{N}{1}$ | 1 |
|  | $P_h$ 1.0 | $\frac{N}{10}$ | $\frac{1}{10}$ |
|  | $P_h$ 2.0 | $\frac{N}{100}$ | $\frac{1}{100}$ |
|  | $P_h$ 3.0 | $\frac{N}{1000}$ | $\frac{1}{1000}$ |
|  | $P_h$ 4.0 | $\frac{N}{10000}$ | $\frac{1}{10000}$ |
|  | $P_h$ 4.5 | Color change—methyl orange | $\frac{3.2}{100000}$ |
|  | $P_h$ 5.0 | $\frac{N}{100000}$ | $\frac{1}{100000}$ |
|  | $P_h$ 6.0 | $\frac{N}{1000000}$ | $\frac{1}{1000000}$ |
|  | $P_h$ 6.8 | Color change—litmus | $\frac{1.6}{10000000}$ |
| Neutral: | $P_h$ 7.0 | $\frac{N}{10000000}$ | $\frac{1}{10000000}$ |
| Alkaline: | $P_h$ 7.5 | Blood | $\frac{3.2}{100000000}$ |
|  | $P_h$ 8.0 | $\frac{N}{1000000}$ | $\frac{1}{100000000}$ |
|  | $P_h$ 8.5 | Color change—phenolphthalein | $\frac{3.2}{1000000000}$ |
|  | $P_h$ 9.0 | $\frac{N}{100000}$ | $\frac{1}{1000000000}$ |
|  | $P_h$ 10.0 | $\frac{N}{10000}$ | $\frac{1}{10000000000}$ |
|  | $P_h$ 11.0 | $\frac{N}{1000}$ | $\frac{1}{100000000000}$ |
|  | $P_h$ 12.0 | $\frac{N}{100}$ | $\frac{1}{1000000000000}$ |
|  | $P_h$ 13.0 | $\frac{N}{10}$ | $\frac{1}{10000000000000}$ |
|  | $P_h$ 14.0 | $\frac{N}{1}$ | $\frac{1}{100000000000000}$ |

**Indicators.**—An indicator is a substance which in the presence of other substances assumes a distinct color varying with the amount of acid or alkali present in this case; or which changes sharply from one color to another as the reaction of the solution which contains it is changed.

The chief indicators used in bacteriological work are litmus, phenolphthalein, the newer indicators such as brom thymol blue, brom cresol purple, phenol red, etc. (see p. 104); the anilin dyes and acid dyes as used by Andrade and Endo respectively (p. 106); and insoluble carbonates (p. 107).

Indicators are used in three ways:

1. To determine reaction of culture media in order to adjust it during the process of preparation.
2. To determine the end reaction of culture media after bacterial growth, for example in toxin production.
3. To be incorporated in culture media to indicate changes in reaction due to growing bacteria.

1. **Adjustment of Reaction.**—In the determination of the true reaction and careful adjustment of culture media lies much of the success of media-making. It is necessary here to take into account the fact that media which are adjusted to a definite reaction before sterilization are found frequently to have changed somewhat during this process of heating. This is true particularly of those media made

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[1] A. L. Wachter: Personal Communication.

[2] The $P_h$ values given as the points of color change for the indicators recorded are, of course, only approximations. In the chart, as a whole, absolute accuracy has been sacrificed for simplicity of presentation.

[3] $C_h$ is the symbol used for "concentration of hydrogen."

with fresh meat as a basis (Anthony and Ekroth.[1]) The effect of the heat applied in sterilization, especially that of the autoclave, tends to render them still more acid, due to the dissociation occurring in some of the ingredients. After a little experience one can learn to make allowance for this change in reaction, and, by the correct overadjustment during the preparation of a medium, obtain the final reaction desired after sterilization. For accurate work this final reaction should be determined on a sample of the finished product.

LITMUS.—Litmus was the first indicator used in bacteriological work and has given valuable service for its change of color (from red in the presence of acid to blue in the presence of alkali) is about $P_h6.8$, very near that of true neutrality or $P_h7$ (p. 100). However litmus is inaccurate in H-ion concentration measurements, partly because it is not a definite compound and partly because azolitmin, its chief component, even though chemically pure, varies in its color reactions according to the material present in the solution being tested.

It is used chiefly as *litmus paper*, red and blue. By dipping these strips of paper in the medium to be tested we can determine roughly the reaction and adjust it either to neutral or slightly alkaline to litmus by adding a 4 per cent. solution of sodium hydroxide and retesting with the litmus paper. The red strip should turn slightly blue or the blue strip just a tinge less blue. *Litmus solution* (p. 106) may be used by adding 0.5 c.c. of a 5 per cent. solution to 5 c.c. of medium in a test-tube. Proceed as in Titrimetric Method given under Phenolphthalein (see below).

PHENOLPHTHALEIN—Phenolphthalein gives good results within the $P_h$ limits, between which its color changes, delicate pink to red, take place (about $P_h8$ to $P_h10$). As this is considerably on the alkaline side of true neutrality, it is colorless in acid solutions. Its use involves the operation called *titration*.

The materials necessary are a burette held in a clamp on a ring-stand, casserole and stirring rod, normal and twentieth normal sodium hydroxide and *phenolphthalein solution* (5 grams of the commercial salt to a liter of 50 per cent. alcohol.

Two methods may be used: (*a*) Room temperature (standard method) and (*b*) boiling temperature. The former should be used when the medium to be tested has been heated previously to the boiling-point during its preparation (p. 95, under Method II); with this method the reaction of a medium is set at a temperature more nearly approximating that at which it will be used, 37° C. of the incubator.

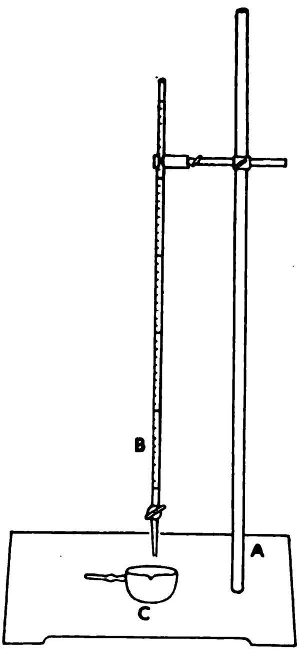

FIG. 25.—*A*, ring stand; *B*, burette; *C*, casserole.

It is convenient, however, to use method (*b*) (boiling temperature) when titrating meat juice which has not been heated above 50° C. (p. 95, under

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[1] Jour. Bacteriol., 1916, i, 209.

Method I) for dissolving the added peptone and salt. Here the boiling temperature method approximates roughly the future conditions when the medium is boiled and sterilized. It has several serious objections, especially when it is misapplied: (1) The indicator phenolphthalein is less sensitive at the boiling-point and therefore less accurate. When a finished medium, prepared from fresh meat according to Method II (p. 95) and adjusted wrongly by the boiling temperature method, is tested by the room temperature method it will be found anywhere from 0.4 to 0.8 more alkaline than indicated by the boiling method. (2) The oft-quoted reason of ridding the mixture in the casserole of carbon dioxide is fallacious. If the sample is taken from a kettleful of medium boiling on the stove it contains little or no $CO_2$. The use of freshly boiled and cooled distilled water for the dilution (see below) eliminates still further the possible presence of $CO_2$.

*Technic of Room Temperature Method.*—Put 45 c.c. distilled water (see above) in a casserole. Add 1 c.c. phenolphthalein solution and 5 c.c. of the medium to be tested. (When titrating agar, warm the distilled water to 35° or 40° C., not above.) If no pink color is present the medium is acid to phenolphthalein. Read the burette, which contains twentieth normal sodium hydroxide (N/20 NaOH) and record the figures. While the mixture in the casserole is being stirred, run in a little of the solution from the burette very slowly until a faint pink[1] tinge is observed. This color should not disappear on stirring.

Read the burette again and record figures. Determine amount of N/20 NaOH withdrawn from the burette. On this is based the calculation for the adjustment of the reaction of the medium (see below).

*Technic of Boiling Temperature Method.*—Five c.c. of the medium to be tested and 45 c.c. of distilled water are mixed in a casserole and boiled for one minute, then 1 c.c. phenolphthalein solution is added. If no pink color is present the medium is acid to phenolphthalein. While the mixture is still hot N/20 NaOH is added slowly from the burette, as above, until a faint but distinct pink color appears. This color must remain on stirring, otherwise more alkali is needed. From the amount added is estimated the amount of normal N/1 solution of NaOH needed to obtain the desired reaction. For example:

*Calculation.*—Five c.c. of medium required 2.4 c.c. of N/20 NaOH, therefore 100 c.c. (twenty times as much) would require 2.4 c.c. of N/1 NaOH (twenty times as strong); in other words, the medium is 2.4 per cent. acid to phenolphthalein or +2.4 if expressed according to *Fuller's method* or *scale*. Assuming that we desire a reaction of +1 per cent., we must then add 2.4 − 1 c.c. or 1.4 c.c. of N/1 NaOH to every 100 c.c. of medium or 14 c.c. to a liter.

If, on the other hand, the mixture in the casserole should show a pink color when the phenolphthalein is first added the medium is alkaline to this indicator. To determine the degree of alkalinity by the titrimetric method twentieth normal hydrochloric acid (N/20 HCl) is used in a burette and the same procedure followed as given above.

In the calculation if we use 0.5 c.c. of N/20 HCl then the medium is 0.5 (−0.5 per cent.) alkaline to phenolphthalein (*Fuller's scale*) and will require 0.5 c.c. of N/1 HCl for every 100 c.c. of medium or 5 c.c. for each liter. But if we wish the reaction to be + 1 or 1 per cent. acid, we must add 5 c.c. + 10 c.c. or 15 c.c. of N/1 HCl to each liter.

However, the addition of hydrochloric acid to a medium at any time is not at all to be recommended, as it may affect the growth of sensitive organisms. As a matter of fact, few media are naturally alkaline to phenolphthalein before adjustment. Such a condition occurs usually when an incorrect amount of alkali has been added in the attempt to reduce the natural acidity. The reduction of the natural acidity of a medium is the wisest procedure to follow, and it has been found far better to discard a medium which is too alkaline rather than to attempt to correct the error which has been made.

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[1] The pink may be described as a combination of 25 per cent. red and 75 per cent. white as shown by the disks of the standard color top.

*Errors of the Titrimetric Method.*—As stated above, the "acidity" of the medium is based on the amount of N/20 NaOH withdrawn from the burette. In adjusting the reaction of a medium to "neutral to phenolphthalein" (about $P_h$ 8, the point of first color change of this indicator) this procedure is accurate, for in this case the whole amount of calculated N/1 NaOH is added. If, however, we attempt to use the Fuller method or scale and adjust, for example, to +1 or "1 per cent. acid to phenolphthalein" by adding only a part of the calculated amount of N/1 NaOH, we fall into error. To be sure, we have lessened the acidity by adding a certain amount of N/1 NaOH; but, because of the presence of an unknown amount of buffer substances (which combine with a part of the alkali added), we do not know how much acid has been neutralized nor the actual acidity of the medium when we have finished, unless we apply the colorimetric or electrometric method at this point (p. 98).

In other words, the titrimetric method takes into account the total acidity of a solution and gives no data as to the actual acidity at a given time (p. 97).

NEWER INDICATORS[1] (for use in the colorimetric method).—For bacteriological work the most useful of these are:

| | | Range $P_h$. |
|---|---|---|
| Brom cresol purple[2] | yellow-purple . . . . . . . . . | 5.2–6.8 |
| Brom thymol blue[2] | yellow-blue . . . . . . . . . . | 6.0–7.6 |
| Phenol red[2] | yellow-red. . . . . . . . . . . | 6.8–8.4 |

In making up the solutions[3] of these indicators use 0.04 per cent. in 95 per cent. alcohol of the brom cresol purple and brom thymol blue; of the phenol red, use 0.02 per cent.

The materials needed are test-tubes of clear glass and uniform diameter (6 by ½ inches is a convenient size), freshly distilled water (ammonia-free), normal and twentieth normal sodium hydroxide solutions, burette, comparator block (p. 105) and the standard solutions of known hydrogen ion concentration (p. 98), *which must be used for comparison in the same dilution and with the same amount of indicator as the medium being tested.* For practical purposes it is well to have a fixed dilution, for example, 2 c.c. of medium to 8 c.c. of distilled water. Prepare a set of standard solution tubes by adding 8 c.c. of distilled water to 2 c.c. of each one of the standard mixtures (p. 99); then add 0.5 c.c. of the chosen indicator to each tube and cork and label each tube plainly with its $P_h$ value.

*Procedure.*—Put 2 c.c. of the medium to be tested and 8 c.c. of distilled water in each of two test-tubes (if agar is being tested warm the water to 35° to 40° C., not above). To one tube add 0.5 c.c. of indicator, choosing one whose range covers the desired hydrogen ion concentration: for example $P_h$ 7.4. Compare with the standard tube of the desired value ($P_h$ 7.4) containing the same indicator. If the shades of color are not the same and the media tube is found to have a $P_h$ value less than 7.4, add to it N/20 NaOH, drop by drop, from a burette, mixing the contents of the tube after each addition of alkali until the shade of color matches that of the chosen standard tube ($P_h$ 7.4). Although the color of the medium itself is lessened by dilution and the chance of error thus diminished (p. 98), the comparison is best made in the comparator block (p. 105), in which the color of the media tube without any indicator is superimposed on the standard tube.

25 × the amount of N/20 NaOH withdrawn from the burette gives the amount of N/1 NaOH to be added to 1 liter of the medium when 2 c.c. are tested.

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[1] A full list covering the whole range of $P_h$ 1 to $P_h$ 10 will be found in the article by Clark and Lubs (page 98).

[2] In ordering these indicators use the chemical names as follows:
Di bromo ortho cresol sulfon phthalein (brom cresol purple).
Di bromo thymol sulfon phthalein (brom thymol blue).
Phenol sulphon phthalein (phenol red).

[3] Report of Committee on Chart, etc., Jour. Bacteriol., 1918, iii, 2.

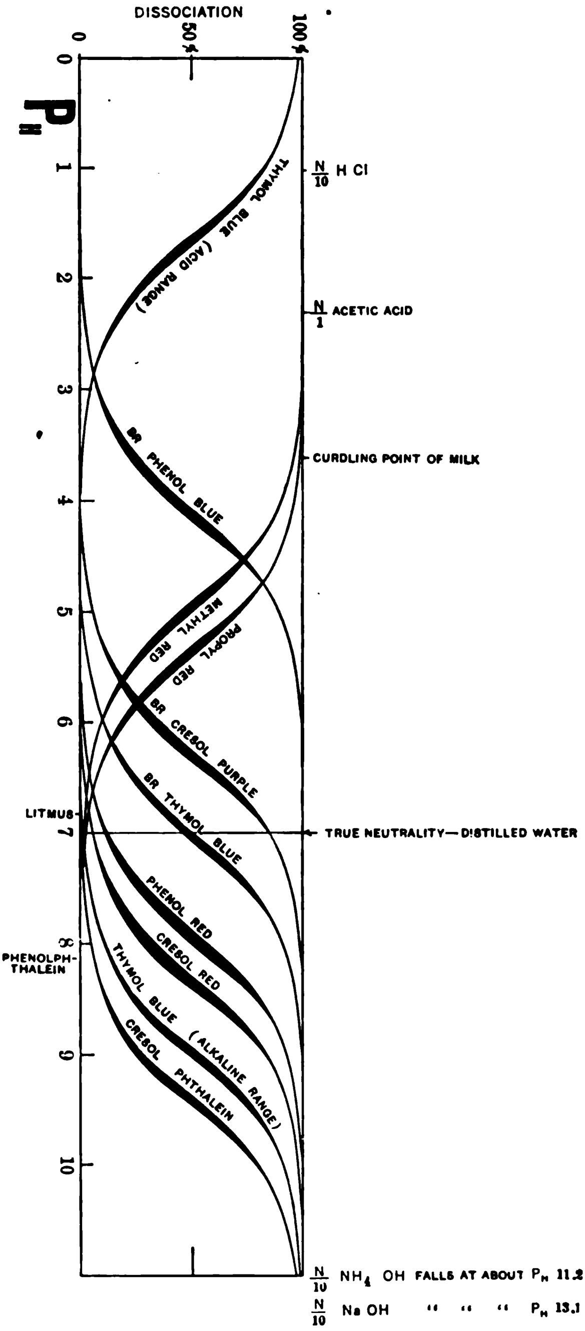

Fig. 26.—Dissociation curves of indicators considered as simple monobasic acids, showing percentage color change with $P_h$. Shaded portions of curves indicate the useful ranges. (Clark and Lub's chart modified.)

Example: If 2 c.c. of medium required 0.3 c.c. of $\frac{N}{10}$ NaOH, 5 c.c. of medium would require 2.5 × 0.3 = 0.75 c.c.; therefore, 100 c.c. (twenty times as much) would require 0.75 c.c. of $\frac{N}{1}$ NaOH (twenty times as strong) and 1000 c.c. would require 7.5 c.c. or 0.3 × 25 = 7.5 c.c.

*Comparator Block.*—The use of a comparator block is very helpful, especially when the material to be tested is either colored or turbid. The block can be easily constructed by boring holes, as in diagram, for the test-tubes in a piece 2 x 4 inches. Slits or other holes are then cut in the sides so that the tubes can be viewed in pairs from the side, and are arranged as follows:

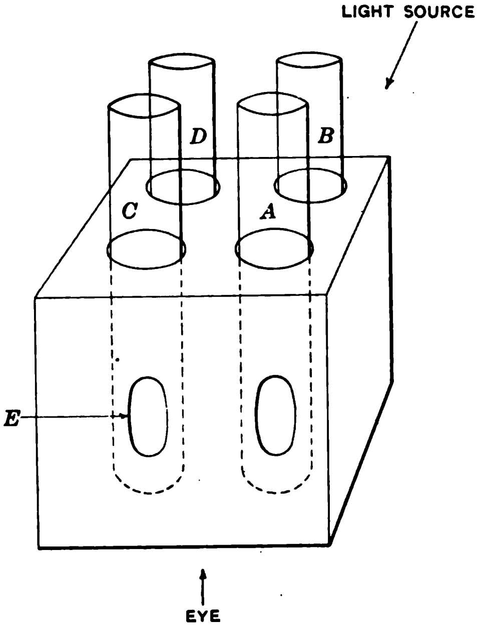

**Fig. 27.—Comparator Block: *A*, medium + indicator; *B*, water; *C*, medium + no indicator; *D*, standard solution + indicator; *E*, slit for observation.**

To prepare a tube of medium for comparison with the Barnett and Chapman tubes (p. 99) add 4 c.c. of distilled water to 1 c.c. of the medium and then 10 drops of the indicator. View this tube with a tube of distilled water to form a pair to correspond with the pairs in the set. A comparator block for this set would require two sets of three holes each in order to view the tubes in threes.

2. **Determination of End Reaction of Culture Media after Bacterial Growth.**—The degree of alkalinity or acidity produced in culture media after bacterial growth is most accurately determined by the electrometric or colorimetric methods. The fallacies of the titrimetric method have been pointed out on p. 103. See also p. 97, actual acidity and total acidity (p. 97).

Either the whole culture or a portion of it may be subjected to the tests as given above.

METHYL RED is used to differentiate between the colon and aërogenes cloacæ groups. The former group gives a distinct red color and the latter a distinct yellow color with this indicator. Prepare the methyl red solution by dissolving 0.1 gram in 300 c.c. alcohol and dilute to 500 c.c. with distilled water. Add 5 drops of this solution to each 5 c.c. of culture.

The culture medium, dextrose phosphate broth, for this test consists of 800 c.c. distilled water, 5 grams of Witte peptone (no other), 5 grams c.p. dextrose and 5 grams $K_2HPO_4$. Heat over steam with occasional stirring for twenty minutes. Filter through paper, cool to 20° C. and dilute to 1000 c.c. with distilled water. Put 10 c.c. in each test-tube. Sterilize in an Arnold sterilizer for twenty minutes on three successive days.

3. **Indicators Incorporated in Finished Media to Indicate Changes in Reaction Due to Growing Bacteria.**—LITMUS (see also p. 101).—Litmus for this purpose is in the form of a solution (see below). The reaction of the medium to which it is added must be neutral (or very slightly alkaline) to this indicator. The disadvantage of litmus is that it may be decolorized by the growth of bacteria which rob it of its oxygen and reduce it to the colorless leucobase. The color will return on exposure to the air. When stab cultures are used it may be necessary to melt the medium and pour it into Petri dishes to get sufficient air to cause the color to return. If the color change of individual colonies is to be observed the diffusion of any acid produced may be prevented by using 3 per cent. agar instead of 1.5 per cent.

*Litmus Solution.*—A purified litmus (Merck or Kahlbaum) which comes in dry form is pulverized and added to distilled water in a 5 per cent. amount. Steam in an Arnold sterilizer for two hours, shaking the mixture every twenty minutes. The solution is then filtered and sterilized preferably in small amounts for convenience as it must be kept sterile. Usually 5 per cent. of the solution is added to a medium, but this may be varied to suit individual preferences. A solution which comes already prepared (Kubel and Thiemann) may be used.

PHENOLPHTHALEIN.—The medium should be faintly alkaline to this indicator and sufficient 1 per cent. alcoholic solution added to give a faint distinct pink color. The color is discharged when acid is produced.

NEWER INDICATORS (p. 103).—For more definite determination of the changes in reaction one or more of the newer indicators should be used. Brom cresol purple, phenol red and cresol red are perhaps the most useful. (It is to be noted that brom thymol blue gives unsatisfactory results in milk.) The choice depends chiefly on the initial reaction of the finished medium. That indicator should be selected whose range either covers the initial reaction (for example, $P_h$ 7) or whose range is slightly to the acid side of the initial reaction: for example, brom cresol purple ($P_h$ 5.2 to 6.8). As the bacterial growth proceeds, changes in the $P_h$ value of the medium may be watched and recorded from day to day.

The above indicators are not reduced by bacterial growth (as are litmus and methyl red) nor do they themselves, in the minute quantities necessary, affect bacterial growth. The amount of indicator used in the medium is not important so long as the color obtained is distinct. A convenient strength has been found to be 1.6 per cent. alcoholic solution. Add 1 c.c. of this to 1 liter of medium. It must be recalled, however, that in making comparisons with the standard tubes (p. 99 and 103), proportionate amounts of the indicator should be present in the standards. These indicators are not affected by sterilization in the autoclave.

Various ANILIN DYES which are reduced to their colorless leukobase by the action of sodium sulphite (Endo) or acid dyes decolorized by sodium hydroxide (Andrade[1]) may be used as indicators. When the sugar is split by the bacteria, aldehydes and organic acids are produced, which cause the color to return. The former act most vigorously on the Endo indicator. (See Typhoid Media.) Media to be used with the Endo indicator should be alkaline to litmus. The Andrade indicator consists of 100 c.c. of a 0.5 per cent. watery solution of acid fuchsin decolorized by the addition of 16 c.c. N/1 NaOH solution. One per cent. is added to the media. The reaction of the medium is adjusted to the indicator so that it is distinctly pink when hot but colorless when cold.

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[1] Jour. Infect. Dis., 1014, xv, 227.

Powdered Insoluble Carbonates may be used in solid plating media. If the powder is evenly distributed throughout the media, acid-producing colonies will be surrounded by a clear area.

In general media containing indicators are sterilized preferably in an Arnold sterilizer. The autoclave may be used in emergencies, but the indicator is somewhat injured.

**Clearing of Media.**—This is done by the coagulation of an albumin by heat (autoclave, Arnold or by boiling). As the albumin coagulates it enmeshes the fine particles floating in the medium and the whole mass is later removed by filtration. The albumin may be either that present naturally, as in meat infusion (soluble albumin, p. 92), or added materials as eggs, either fresh or in the form of dried egg albumen. The hot medium must be cooled to about 50° C. before the egg is added.

If fresh eggs are used one is allowed to each liter. After breaking the egg in a small pan it is mixed with an equal amount of water by means of an egg beater. The mixture is then added to the cooled medium and the whole stirred thoroughly. When dried egg albumen is used 10 grams are added to 20 c.c. of water and allowed to stand overnight to dissolve thoroughly. Add the 20 c.c. to 1 liter of medium as above.

The medium is then heated usually in an autoclave at 10 to 15 pounds pressure for thirty minutes or in an Arnold sterilizer for forty-five to sixty minutes. Boiling over the free flame is not so satisfactory.

**Filtration of Media.**—For *fluids* use filter paper reinforced with a piece of cotton at the neck of a glass funnel. The paper used should be moistened with cold water to prevent the passage of any fats present in the medium.

Media which solidify on cooling (agar and gelatin) are usually filtered satisfactorily through absorbent cotton in a glass funnel kept warm by a hot-water jacket (Fig. 28). However, the use of paper-pulp in a Buchner funnel (p. 108) or of a Sharples centrifuge is desirable at times to produce a very clear medium. On the other hand mere sedimentation may be all that is necessary or advisable. (See Vitamin Agar.)

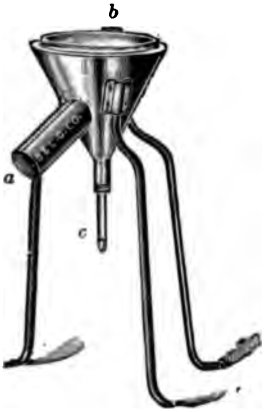


Fig. 28.—Hot-water funnel or jacket: *a*, point heat; *b*, inlet for water; *c*, glass funnel. The water is kept hot by placing flame of Bunsen burner under *a*.

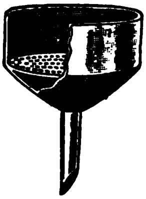


Fig. 29.—Buchner funnel.

**Preparation and Use of Funnels.**—A good grade of absorbent cotton with long fibers is essential so that the usual layer of cotton may be split horizontally. Lay the split portions crosswise in a glass funnel in which has been placed a small square of coarse wire netting, about one-quarter inch mesh, such as wire baskets are made of. This prevents the cotton from jamming in the neck of the funnel and also gives a broad dripping surface. The glass funnel should be placed in a *hot-filtration funnel* (Fig. 28) or kept warm in an Arnold sterilizer.

Moisten the ends of the cotton with some of the medium so they will adhere to the glass, then carefully moisten the rest of the cotton with a dipperful of medium. Add small portions of the medium slowly at first. The coagulated egg and albumins which settle on the cotton act as a part of the filter. Pour back the filtrate several times until it comes through clear. The flasks in which it is received should be warmed by rinsing with hot water. The rest of the medium should be kept hot in the Arnold sterilizer until it is put in the filter.

**Paper-pulp in Buchner Funnel.**—The nine-inch size Buchner funnel is suitable for media in 5- to 10-liter lots. It is used with a suction pump. The ordinary filtration pump (p. 114) which gives about eleven inches' vacuum is sufficient. *Paper Pulp.*—Prepare paper-pulp by soaking scraps of ordinary filter paper for thirty-six to forty-eight hours in hot water in a large wide-mouthed bottle. The paper and water should be in the proportion of 6 sheets of filter paper (20 x 20 inches) to 2½ liters of water. Shake the mixture vigorously at intervals to make the suspension fine and uniform. When ready to prepare the nine-inch funnel, dilute 400 to 500 c.c. of the pulp with about 3 liters of hot water. Cut a piece of surgeons' lint (or cotton flannel) to fit the bottom of the funnel exactly. Rinse the funnel with hot water. Place in it the lint, fleecy side uppermost. Pour in the hot paper-pulp suspension carefully so as to cover the lint with an even layer about one-eighth to one-fourth inch thick. Over this lay a disk of filter paper. Place a 4-liter suction flask under the funnel and apply the suction pump to draw the water into the filtration flask until the pulp is firm, yet somewhat moist. The agar will not go through too dry a filter.

The funnel and paper-pulp must be hot when the agar is poured in carefully and slowly, striking the disk of filter paper which prevents the breaking of the surface of the pulp. Discard the first 100 c.c. of agar which come through for they contain some of the water from the pulp.

Media filtered by this method must *not* have egg added for clearing. It clogs the filter.

The **Sharples Super-centrifuge** works on the principle of the milk separator. The machinery is very complicated, but it gives excellent results.

**Sedimentation.**—With this method the agar medium is allowed to cool slowly, so that the larger particles may settle at the bottom of a straight-sided container. After it has stood overnight the hardened mass is turned out as from a jelly mould and the sediment trimmed away from the clearer portion. The fine particles remain in the medium. By sedimentation, or by use of a Sharples centrifuge, all contact of the medium with paper or cotton may be avoided and the "vitamins" (p. 124) retained.

**Preparation of Media for Use.**—After filtration the medium may be sterilized (p. 110) either in flasks for storage or in various containers suitable for immediate use—such as test tubes and bottles. In filling any container avoid wetting the neck of it or the cotton plug will stick. Use a glass funnel with a stop-cock; or fit rubber tubing, with a piece of glass in one end, on an ordinary funnel and control the outflow with a pinch-cock (Fig. 42).

The amount of medium in a container depends on the use to which it will be put. For the ordinary 15 cm. (six-inch) test-tube a depth of 3 to 5 cm. is sufficient. If plates are to be poured at least 10 c.c. are necessary, but if the medium is to be slanted, 6 to 7 c.c. suffice. The slants may be made either immediately after sterilization or the medium may be remelted and slanted as required.

It is convenient to remelt sterilized media by immersing the tubes, held in a wire basket, in a small kettle of water which should rise above the level of the medium. The water is then brought to the boiling-point and kept there until the medium is melted. The flame should then be lowered and the medium removed as promptly as possible from the influence of the high temperature.

Prolonged or repeated heating of a medium tends to change the reaction and also reduces the solidifying qualities of agar as well as of gelatin. For bottles, flasks or large numbers of tubes the Arnold sterilizer may be used.

To make the slants, place the tubes after sterilization in a row on a table with a glass rod or strip of wood to raise the upper ends. Care should be taken that no slant touches the cotton and that sufficient medium be present in the bottom of the tube ("butt") to prevent the slant from collapsing when the tube is raised. The medium should be well set before the tubes are moved.

**Fermentation Tubes** (Fig. 20).—Fermentation tubes are used for liquid media only.

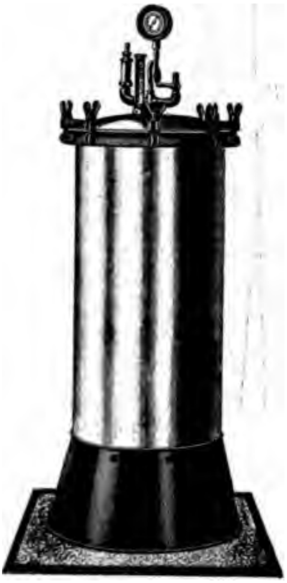


Fig. 30.—Upright type.

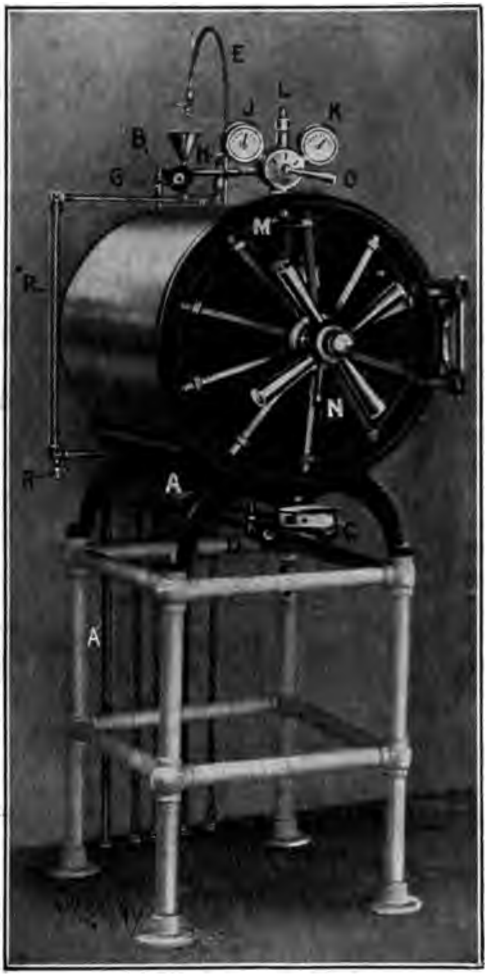


Fig. 31.—Horizontal type.

Figs. 30 and 31.—Autoclaves may be heated by direct application of heat, or when available, by steam under pressure.

In filling types "a" and "b" the tube must be tilted until no air-bubble remains in the closed arm. With type "c" the small inner tube, which is separate, is filled first, then a small amount of medium is run in the large tube. Slip the small tube, with as little inversion as possible, mouth down into the large tube. There should no no air-bubble in the smaller tube. Practice is needed for this operation. Add more medium if necessary to the larger tube and replace plug.

All fermentation tubes must be sterilized in the Arnold sterilizer (p. 113).

**Sterilization of Media.**—Sterilization may be accomplished by *heat*, *filtration* or by the use of *chemicals*.

**Heat.**—The heat may be applied in one of several ways: (1) Steam under pressure (autoclave); (2) streaming steam (Arnold); (3) water-bath. Methods (2) and (3) are usually known as intermittent ("fractional") sterilization.

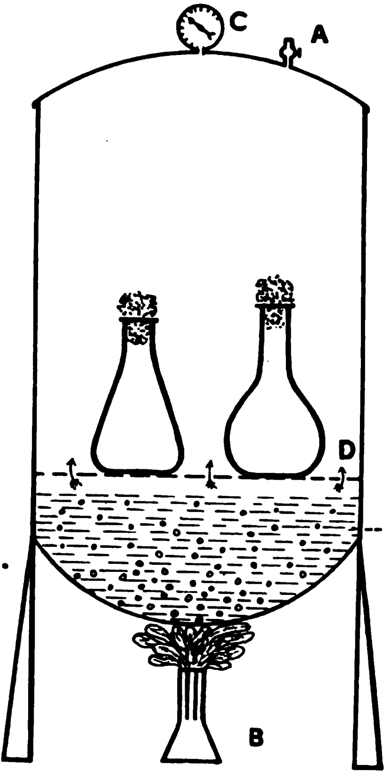


FIG. 32.—Cross-section of simple upright autoclave. *A*, pet-cock or vent; *B*, source of heat; *C*, dial; *D*, steam.

1. **Steam under Pressure.**—The sterilization is accomplished by means of an autoclave, various forms of which are available (Figs. 30 and 31). They may be heated either by gas or steam from the boiler-room. All air in the chamber should be displaced by the steam before closing the pet-cock or vent in order to avoid air-pockets which interfere with thorough sterilization. In the simplest form (Figs. 30 and 32) the water in the bottom is brought to the boiling-point after the lid is clamped down. The displacement of any air in the chamber is accomplished by allowing the vent to remain open until steam has issued for a minute or two. The steam is then brought up to the desired pressure, as shown by the pressure-gauge, by continued heating. A safety valve set to the required pressure releases a portion of the steam when that pressure is reached. In the more complex type (Figs. 31 and 33) the water in the surrounding jacket is heated and part of it converted into steam until the pressure in the jacket exceeds by several pounds the pressure desired in the chamber,

By a system of valves the steam from the jacket is then allowed to fill the chamber and, after displacement of the air as above, the vent is closed and the pressure in the chamber reaches the required point. This pressure is controlled by a safety valve (*L*, Fig. 31).

At the end of the "run" the steam supply is shut off and the pressure allowed to decrease gradually by opening the vent a very little. Too rapid a reduction of the pressure will cause the media to boil up and wet or even blow out the plugs.

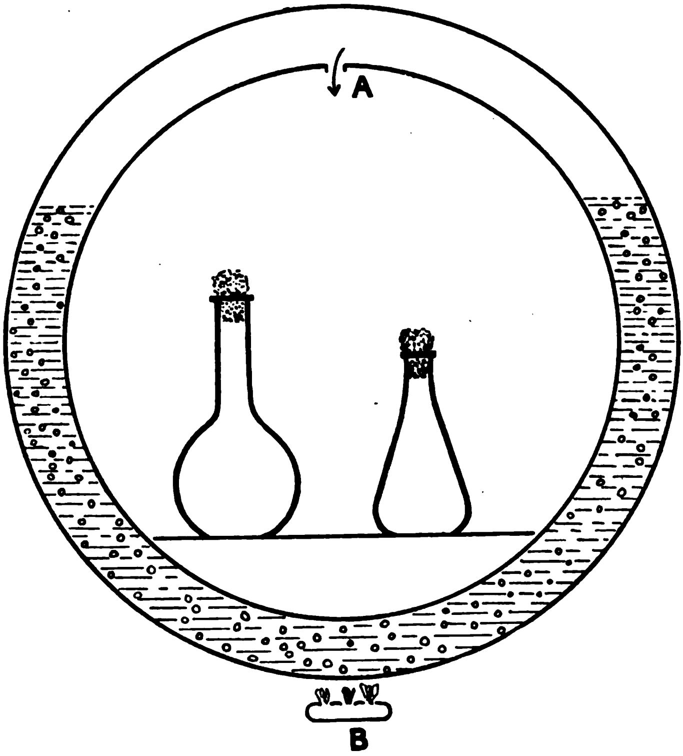


FIG. 33.—Diagram of inner construction of horizontal autoclave. *A*, passage for steam from jacket to chamber; *B*, source of heat if gas is used.

The pressure and time employed depend on conditions. As a rule, 15 pounds' pressure is employed and twenty minutes is sufficient for media in test-tubes. Flasks should be heated one-half to one hour, depending on their size. Lower pressures are used at times when overheating is undesirable, and the heating repeated on two successive days. The heating should be calculated from the time the desired pressure is reached. The temperature will vary with the pressure thus:

| | | | | |
|---|---|---|---|---|
| 5 pounds' pressure | = | temperature | . . . . . . . . . | 108.8° C. |
| 10 " " | = | " | . . . . . . . . . | 115.6° C. |
| 15 " " | = | " | . . . . . . . . . | 121.3° C. |

Since the pressure in the autoclave may fluctuate it is desirable to use a recording pressure-gauge which shows graphically on a chart the period of the run and the pressure maintained (Fig. 34). These charts may be dated and filed for reference.

Agar media must be in the liquid state when put in the autoclave for sterilization.

2. **Streaming Steam.**—Streaming steam is applied by means of an Arnold sterilizer (Figs. 35 and 36). By this means a temperature of 100° C., or close to it is applied to media containing special sugars, etc. This heating must be repeated on three or more successive days, the daily period depending on the size of the container; test-tubes of media require twenty minutes, whereas 1- to 2-liter flasks require forty-five to sixty minutes to allow the heat to penetrate. With agar media the period is timed from the minute the agar is wholly melted.

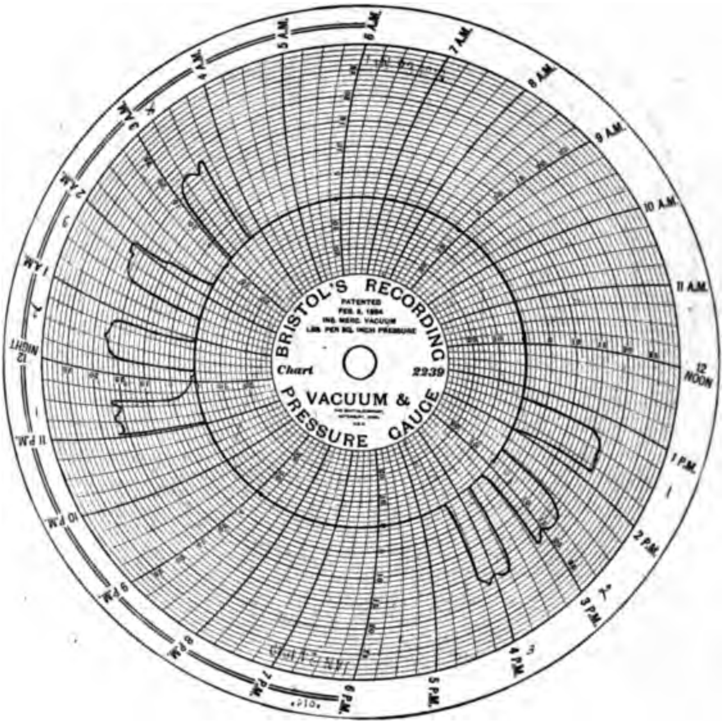


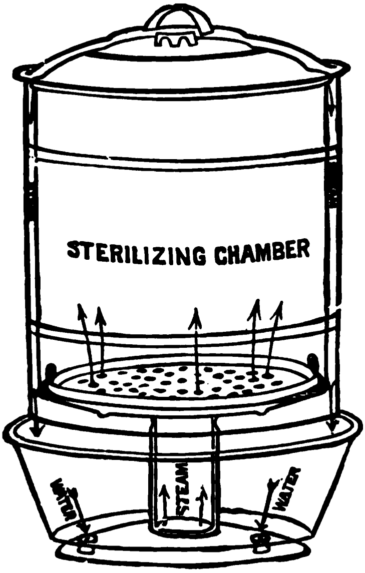
FIG. 34.—Recording chart of autoclave.

3. **Water-bath.**—In this method temperatures of 60° to 80° C. are applied on successive days to such substances as blood serum, transudates from body cavities (*e. g.*, ascitic fluid) etc.

As stated above, methods (2) and (3) are known as intermittent (or "fractional") sterilization.

The principle underlying fractional or intermittent sterilization is as follows: All bacteria when free from spores are killed by exposure for one or two hours to only 60° to 80° C. heat. If the material is left overnight at about 22° C., spores, if present, will develop into bacteria and be destroyed by the second heating. Some of the bacteria may produce spores before the second heating or some of the spores may be slow in developing and escape. For this reason a third heating, or with the low temperatures, five or six heatings on successive days may be necessary.

When method (3) is applied to albuminous material before coagulation, such as sera, tissue extracts, etc., the temperature selected should be below the coagulation-point of the material, usually about 60° C. All material to be treated in this manner should be as free from contamination as possible. The method has its best application to originally sterile material which, because of necessary manipulation may have become slightly contaminated. The heating may be done in a water-bath or in a water-jacket oven.



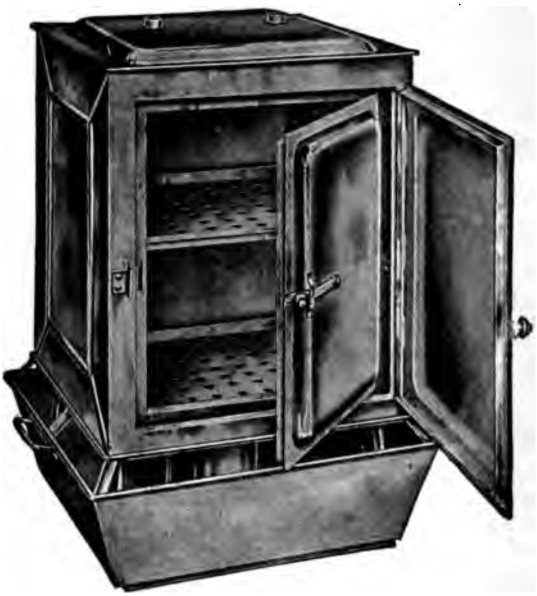


Fig. 35 Fig. 36

Figs. 35 and 36.—Arnold steam sterilizer. Two types.

**Filtration.**—This method is resorted to when the application of heat will injure the solution, as in the case of some sugars, or when the necessary amount of heat would cause coagulation, as in the case of tissue extracts or serum. Various types of filters made of unglazed porcelain or compressed diatomaceous earth are used. Types of these filters are the Berkefeld, Pasteur, Chamberland and Doulton filters. The bacteria are held back, because of the fineness of the pores. Various grades of fineness are procurable in some brands, and on this depends the rapidity of filtration. The filter candle and all attachments with which the fluid will come in contact after passage through the candle must be sterile. The glassware can be sterilized in the hot-air oven and the filter candle and rubber connections can be sterilized by boiling for one hour or by steam under pressure. (See Autoclave.) After use the filter candle should be freed of all soluble material, especially coagulated matter, by running through it an abundance of clear water. If used for infective matter, it can then be sterilized by boiling. In any case the surface should be lightly scrubbed with a fine brush after use.

A new filter should be cleansed before use by filtering clear water. It should then be placed in cold water and boiled thoroughly.

After continued use the candles gradually become clogged. They can be renewed to some degree by careful heating to glowing in an oven. This is apt to produce fissures, hence the filter should be retested before use.

In any case the permeability of the filter should be tested before use by filtering a broth culture of some small microörganism, such as Bacillus prodigiosus. Before filtration the fluid to be filtered should be rendered as clear as possible by filtration through paper or if necessary through paper-pulp.

Coarse filtration is accomplished by using paper-pulp or sand. The paper-pulp is prepared by soaking torn-up filter paper in water and then placing a layer in a porcelain filter, over a perforated porcelain plate, draining off the water and packing the layer tight. The depth of the layer will depend on the fluid to be filtered, its density and the fineness of the particles to be removed.

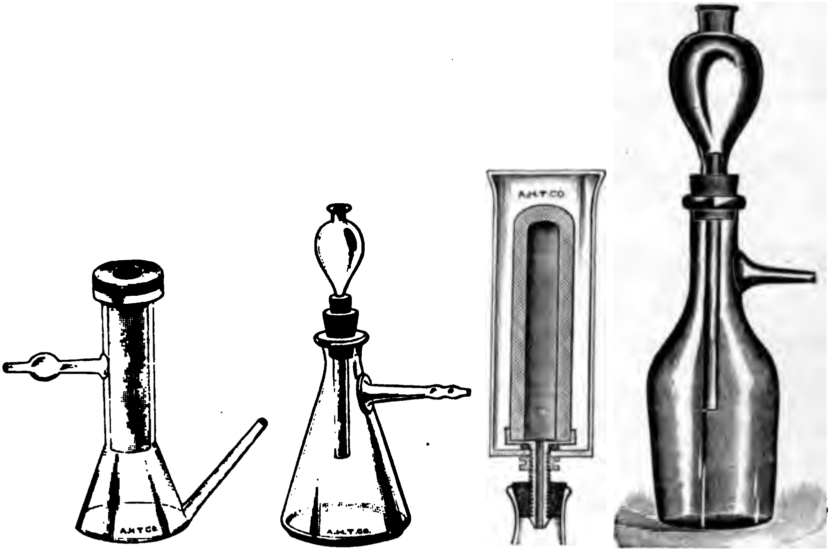


FIG. 37 FIG. 38 FIG. 39 FIG. 40
FIGS. 37, 38, 39 and 40.—Types of filters.

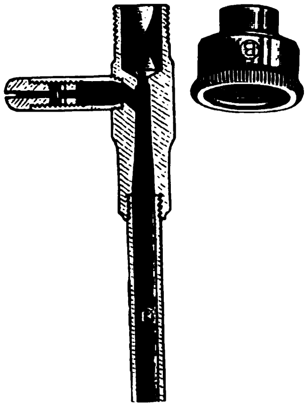


FIG. 41.—Filter pump for attachment to water faucet.

This should be fastened in the neck of a filtering flask and suction applied to hasten the filtration. Similar results may be obtained by filtration through sand.

The filtration through a filter candle (the size selected will depend on the amount to be filtered) is accelerated by the use of suction or pressure. Suction may be secured by the use of a filter pump attached to the hydrant or other types of suction apparatus. Pressure may be obtained by air under pressure from an installed system or by a hand pump and cylinder to equalize the pressure. A manometer is employed to determine the pressure. The accompanying figures show the method of setting up the apparatus (Figs. 37 to 41).

**Chemicals.**—Chemicals such as chloroform are used occasionally, especially for the preservation of sera which may have suffered slight contamination.

**Storage of Media.**—After sterilization media may deteriorate for two reasons—contamination and drying. Contamination is due frequently to the penetration of the stopper by moulds. This may be prevented by capping the container with filter paper before sterilization. Rubber tissue tied over the neck of a flask, or rubber caps made for the purpose are also useful. These are applied after sterilization. A small pledget of cotton moistened with bichloride of mercury solution may be placed between the cap and the stopper to discourage the development of any moulds which may have adhered during the manipulation. The neck of the flask should be wiped free of the bichloride before pouring out the medium.

Drying may be avoided by capping as above or by sealing with paraffin or sealing wax. The plugs of tubes are dipped in hot paraffin. With flasks the plug is cut off, pushed in slightly and the melted paraffin poured on top. The paraffin should be nearly ready to set so that it will not run through the plug into the medium.

It is well to keep most media in a dry refrigerator at 40° C. to 45° C.

## Formulæ and Uses of Culture Media.

**Basic Liquid Media.**—Meat infusion (nutrient) broth and its variant beef extract have been given on p. 95.

**Basic Solid Media.**—**Gelatin Media.**—**Meat infusion gelatin** may be prepared in two ways: 1. To 1000 c.c. meat juice (p. 95 Method I) add gelatin, 10[1] grams together with peptone 10 grams, sodium chloride 0.5 gram. Follow the procedure of Method I. The coagulation of the albumins present in the meat juice clears the medium. Filter through cotton (p. 107), tube and sterilize[2] in the Arnold sterilizer twenty to thirty minutes on three successive days.

2. To 1000 c.c. *meat infusion* (p. 95, Method II) add peptone 10 grams, sodium chloride 0.5 grams. Heat almost to the boiling point, add the gelatin, 10[1] grams, and dissolve with as little heating as possible. Set reaction (p. 100) neutral to litmus (or about $P_h6.8$ or $P_h7$.) Cool to 50° C. and clear with egg (p. 107) by heating for forty-five minutes in an Arnold sterilizer. Filter, tube and sterilize as above.

**Beef Extract Gelatin.**—Add 10[1] grams of gelatin to beef extract broth (p. 96). The gelatin dissolves readily at the boiling-point. Proceed as in meat infusion gelatin, second way.

**Agar Media.**—**Meat Infusion Agar.**—Any one of several methods may be used.

1. The simplest is to add the agar 1.5[3] per cent. to nutrient broth prepared by either Method I or Method II (p. 95). Dissolve the agar either by heating in the autoclave at 10 to 15 pounds for thirty minutes or by boiling over the free flame. In this case the water lost by evaporation should be made up by adding hot water. Set reaction (p. 100). Cool to 50° C. and add one egg for each liter (4 eggs for 5 liters). To clear (p. 107) heat in autoclave at 10 to 15 pounds for thirty minutes or Arnold sterilizer for one hour. Test the reaction and adjust if necessary. If more than 0.2 per cent. normal soda is required per liter; heat again for ten minutes to throw down any precipitate. Filter through cotton (p. 107). Fill tubes or flasks and sterilize at 15 pounds' pressure for thirty minutes.

2. The agar may be added to the meat juice (p. 95, Method I) after the peptone and sodium chloride have been dissolved. Proceed as above.

3. Add the agar to *meat infusion* (p. 95, Method II) together with the peptone and sodium chloride. Proceed as above under (1).

4. Double strength method: Make double strength meat juice (p. 95, Method I) or meat infusion (p. 95, Method II). Add double the amounts of peptone and salt. Set the reaction. To an equal quantity of water add double the amount of agar and dissolve it. Cool the agar below 50° C. and mix the two solutions. Test the reaction and adjust if necessary. Proceed as directed above under (2) or (3) depending upon the choice made as to meat juice or meat infusion.

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[1] Use 12 grams in warm weather.

[2] Avoid overheating and additional sterilization as the medium will not set if heated too much.

[3] Two per cent. is better if other substances such as blood or serum are to be added after sterilization.

**Beef Extract Agar.**—Beef extract agar may be made in several ways:

(1) Add 1.5 per cent. agar to 1000 c.c. of meat extract broth. Proceed as in meat infusion agar under method (1).

(2) Weigh out the ingredients as for beef extract broth and add 1.5 per cent. agar. Dissolve the whole by placing the mixture in autoclave, as above, or by boiling over the free flame. Proceed as in meat infusion agar under (1).

(3) Double Strength Method.—Make a double-strength beef extract broth. To an equal amount of water add double the amount of agar. Dissolve the agar, cool to 50° C. and mix the two solutions. Proceed as in meat infusion agar under (1).

**Semisolid Media.**—Various types of semisolids are in use. The amount of agar will vary according to the use to which it is put. For slants, 0.75 per cent. to 1 per cent. of agar is necessary; for stab cultures, 0.5 per cent. or even less is sufficient. If the agar is to be diluted by the addition of serum or other enriching substance, this must be allowed for in preparing the medium. The following is an example (*North*): Meat infusion (1 pound of meat to 500 c.c. of water) is warmed and 20 grams of gelatin and 20 grams of peptone dissolved in it. In 500 c.c. of water dissolve 10 grams of agar and cool to below 50° C. Mix the two, adjust the reaction, and heat to coagulate the albumin and clear the medium. Readjust the reaction and reheat if necessary; filter.

A very satisfactory medium can be made by simply using 0.5 per cent. of agar instead of the usual 1.5 per cent. employed. This can be diluted by the addition of one-third of its bulk of an enrichment fluid and will still set sufficiently for stab cultures. Nutrose (1 per cent.) may also be added (p. 124).

**Digestion Products in the Preparation of Media.—Martin's Peptone Solution.**[1]—Five pigs' stomachs cleaned, fat removed and finely minced. (A number of stomachs should be used to equalize the peptone content; in this way an almost average composition in peptone is obtained.) Mixed stomachs (minced) 200 grams, hydrochloric acid (pure) 10 grams, water (at 50° C.) 1000 c.c. Keep at a temperature of 50° C. for twenty to twenty-four hours in a glass or porcelain vessel, *not* enamel. It is most important not to allow peptone to come in contact with any metal until it is neutralized. Heat to 80° to stop digestion. Pass through a layer of absorbent cotton. (At this point it can be stored without sterilization.) Heat filtrate to 70° C. and neutralize to litmus at this temperature. Sterilize in autoclave at 10 pounds' pressure for fifteen to twenty minutes and store away.

**Martin's Peptone Broth.**—To meat infusion add an equal amount of Martin's peptone which has been neutralized. The mixture is best made with both the peptone and broth at 70° C. Titrate (room temperature method) and set to desired reaction. Autoclave for fifteen minutes to clear. Titrate again and set reaction as before. Filter through paper and cotton and sterilize in autoclave at 15 pounds' pressure for one-half hour.

**Digested Meat Media.**—Meat, etc., digested by pancreatin, trypsin and other ferments.—Hottinger[2] makes the statement that the greater part of the nutritive elements of meat are lost in the usual process of making broth for laboratory use. To avoid this, a process of slow digestion is recommended, with the addition of pancreatin, and it is claimed that in this way a much more favorable medium can be obtained for the growth of bacteria, with so much peptone developed as a result of the meat digestion, that no addition of commercial peptone is required. Sodium chloride is also omitted.

**Hottinger Broth** (slightly modified).—Use 750 grams of meat to 1500 c.c. water. The meat, carefully freed from fascia, is cut in finger-thick pieces. Heat the water to boiling. Drop in the meat, piece by piece, stirring constantly. Boil up strongly and take from fire. Take out meat and put through chopping machine. Cool water to 37° C. and to it add sodium carbonate, 1.5 grams per liter. Put chopped meat in flasks (2-liter, Erlenmeyer) 550 grams per flask. Add the water (37° C.) to flasks, filling them up to narrow neck. To each flask add: pancreatin 3 grams, chloroform 10 c.c., toluol 10 c.c.

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[1] Ann. de l'Inst. Pasteur, 1898, xii, 38.

[2] Centralbl. f. Bakteriol., 1912, lxiii, 178.

Cork tightly and shake well. Incubate at 37° C. overnight. Shake next day and add more pancreatin unless the fluid shows a yellow color and particles of meat look smaller, showing that digestion is taking place.

The process of digestion should continue for four or five days at room temperature or for two or three days in incubator, shaking well each day. At the end of this time the meat has become a finely divided mass, giving off a very offensive odor. According to Hottinger the medium may be stored in the ice-box at this point without heating again, after testing with litmus paper and acidifying with a slight amount of dilute HCl, if found to be alkaline. In our experience this plan has not worked well, and we have found it best to proceed at once as follows:

Decant liquid through cheese-cloth. Add an equal amount of water to the residue in flask. Shake well. Allow meat to settle and again decant. Finally place meat on cheese-cloth and allow to drain. Boil the filtrate for a few minutes, then filter through absorbent cotton and paper until clear. Store in flasks as stock broth after autoclaving at 15 pounds' pressure for one-half hour.

This stock can be diluted for use as desired, according to Hottinger ten, twenty or more times. Diluted one-half (1 part stock broth, 1 part water) this broth gives excellent results with the ordinary laboratory organisms. The diphtheria bacillus, however, does not grow well.

**Hottinger Agar.**—Add sufficient water to Hottinger stock broth to make the required dilution. Add agar 1.5 per cent. and proceed as usual.

This agar made with Hottinger broth, 1 to 1 dilution, gives good results in successive slant agar cultures of the usual laboratory organisms with the exception of the gonococcus and the diphtheria bacillus.

**Broth Prepared with Trypsin.**—Use 300 to 500 grams of meat to 1 liter of water to which 0.4 per cent. sodium carbonate has been added. Soak overnight, then heat to 80° C. Cool to 38° C. and add 15 c.c. of liquid trypsin. Keep at 38° C. for five hours, stirring frequently. If kept overnight at this temperature, add toluol 10 c.c. (or thymol crystals). Then add hydrochloric acid (normal) to neutralize. Boil seven minutes, strain, set reaction. Boil one-half hour, filter and sterilize.

**Peptic Digest Broth of Liver, Beef or Human Placenta.**[1]—1. Wash, clean and mince finely 5 or more large pigs' stomachs. Mince an equal amount of clean pigs' or beef liver, cheap fat-free beef, placenta or blood clots.

2. Mix in the following proportions: Minced stomachs, 400 grams; minced liver, beef, placenta, 400 grams; HCl, 40 grams; Tap water at 50° C., 4000 grams; and keep mixture at 50° C. for eighteen to twenty-four hours. (Use glass or porcelain receptacles, *e. g.*, museum jars in electrically regulated water bath).

3. Make biuret[2] and tryptophan[3] tests.

4. Transfer to large bottle and steam for ten minutes at 100 °C. to stop digestion. Strain digest through cotton or preferably store overnight in ice-box and decant after twenty-four hours.

5. Warm the filtrate, or decanted digest, to 70° C. and neutralize with sodium carbonate (twice N/1 Sol.) to litmus at this temperature.

6. Sterilize (if not to be used at once) in the autoclave at 10 pounds' pressure for fifteen minutes or for thirty minutes at 100° C. on two successive days and store away.

Stock Digest may be used for: Plain digest broth; sugar-free digest broth; stock digest agar; tryptonized digest broth; sugar-free tryptonized digest broth.

The liver digest agar is especially satisfactory as a substitute basis for the colon-typhoid media which are discussed on p. 125.

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[1] Stichel and Meyer: Jour. Infect. Dis., 1918, xxiii, 78.

[2] To 5 c.c. of filtered digest, add 0.1 c.c. 5 per cent. solution $CuSO_4$; mix and then add 5 c.c. N/1 NaOH. Pink color indicates complete peptonization.

[3] To 10 c.c. neutralized and filtered digest add slowly bromin water until the maximum purple coloration is reached.

**Dilutions of Media.**—Berry[1] has found that good results are obtained in milk plates with a medium containing the usual amount of agar and lessened amounts of meat extract, peptone and salt. With the progressive dilution of the medium there is, at first, an increase in the number of colonies, but beyond a certain point they tend to become smaller and less distinct. On agar made with a dilution of one-twelfth the usual amount of meat extract, peptone and salt, the colonies are practically identical in number and appearance with those on the standard agar.

Still better results for milk work are given by agar made with a 1 to 15 dilution of Hottinger stock broth (p. 116).

**Used Agar.**—It has been found by Berry of this laboratory that veal agar which had been used in Blake bottles for preparation of antigens and vaccines in large quantities, can be used a second time for culture purposes. After the surface growth is removed by scraping and washing with salt solution or distilled water, the agar is melted and the different lots poured together. The agar is then made neutral to phenolphthalein, tubed, sterilized and again planted with various organisms.

Except for a loss of transparency, which could probably be restored by fresh clearing and filtering, the previous use of the agar seems in no way to affect its value as a culture medium. Typhoid, paratyphoid, coli, staphylococcus, streptococcus, pneumococcus, gonococcus, diphtheria and other varieties of ordinary laboratory organisms grow as vigorously as on new agar, and in some cases the growth is heavier. Abundant cultures of amebæ are also obtained on used agar.

**Media with Chief Ingredient other than Meat.**—**Peptone Solution** (Dunham's).[2]—Water 1000 c.c., peptone 10 grams and sodium chloride 5 grams. Dissolve by heating and filter through filter paper. Reaction should be about $P_h7$.

**Milk.**—The milk used must be unpasteurized and as fresh as possible, preferably the best grade obtainable. Steam the milk in the Arnold sterilizer for a half-hour and place on ice for several hours or overnight to allow the cream to rise. By means of a siphon remove the milk from below the cream layer. The milk may be tubed or litmus solution added (*litmus milk*). If a good grade of milk is used no change of reaction is necessary; if acid, sufficient sodium hydrate solution should be added to render it slightly alkaline to litmus; if very acid, the milk should be discarded.

**Whey Broth.**—Milk (unpasteurized), 3 quarts. Let stand overnight and take off cream in morning. Heat milk on stove and stir until ready to boil. Add 6 c.c. glacial acetic acid (0.2 per cent.) to coagulate the casein. Strain through cheesecloth. Add peptone, 2 per cent.; sodium chloride, 0.5 per cent. Boil, stirring to dissolve peptone and salt. Titrate and adjust to 0.5 per cent. acid to phenolphthalein. Add white of 3 eggs to clear after cooling to 45° C. Filter through cotton and paper. Sterilize in autoclave at 15 pounds for thirty minutes on two consecutive days.

**Nitrate Broth.**—Water 1000 c.c., peptone 1 gram, and nitrate-free potassium nitrate 0.2 gram.

**Egg Media.**—Egg media are usually coagulated and therefore solid. Use fresh-laid eggs, as they are free from bacteria.

**Plain Egg** (*Dorset*[3]).—The eggs are thoroughly cleansed with water of any adherent dirt and then washed with 5 per cent. carbolic solution and allowed to dry partially. The ends are then gently dried in the flame and pierced with sharp forceps which have been flamed. The hole at one end should be about ⅜ inch in diameter and the membrane broken; at the other end which is to be blown into, the hole should be smaller and the membrane left unbroken, if possible. The eggs are then blown into a sterile Erlenmeyer flask, the blowing being done from the cheeks. To the egg is then added water (10 per cent. by volume of the weight of the eggs). This is mixed by twirling the flask or by gently stirring with a glass rod. Bubbling must be avoided. The mixture is then filtered through cheese-cloth by gravity and tubed. (See Apparatus, Fig. 42). The tubes are then slanted and coagulated by heating to 70° C. for two or two and a quarter hours on two successive days. No further sterilization is employed. The medium is incubated to test its sterility.

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[1] Coll. Studies, Dept. Health, City of New York, 1913–1915, viii, 288.

[2] Am. Jour. Med. Sc., 1893, cv, 73.

[3] Am. Med., 1902, iii, 555.

**Glycerin Egg** (Lubenau).[1]—Ten eggs are blown into a flask and 200 c.c. of glycerin broth (5 per cent. glycerin, neutral or slightly alkaline to litmus, about $P_h$ 6.8 to 7) added. The further preparation is the same as the preceding.

If the medium requires it, a drop or two of water can be added after coagulation to supply the necessary moisture. To conserve the moisture the plugs should be paraffined or cut off and burned and a charred cork used to seal the tube. (See also Anilin Dyes in Media.)

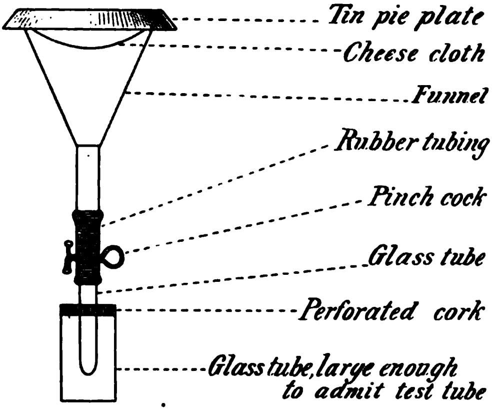

FIG. 42.—Straining and filling apparatus. The lower tube is plugged with cotton and the top wrapped. It is necessary to loosen the pinch-cock before heating. The whole is sterilized in the autoclave. (Krumwiede after Masson.)

**Potato Media.**—Use large white potatoes and scrub them thoroughly under running water. Cylinders are then cut by means of an apple-corer (Fig. 43). Cut the skin off the ends of the potato cylinder and by an oblique cut make wedge-shaped pieces (Fig. 44). A good butt should be left or the pieces will curl on sterilization; curling occurs also if the pieces are too small and thin. While the potatoes are being prepared they should be kept under water to avoid discoloration of the cut surfaces. The reaction of potatoes is acid. This must be corrected by soaking them either in running water overnight or in a 1 to 1000 solution of sodium carbonate for several hours. The pieces are then placed in test-tubes, one inch or more in diameter (Fig. 45); a little water may be added to avoid drying. Sterilize in the autoclave at 15 pounds' pressure for thirty minutes.

**Glycerin potato** for the cultivation of tubercle bacilli should be cut as above, soaked overnight in 1 to 1000 sodium carbonate solution, drained, and covered with a 5 per cent. glycerin solution for twenty-four hours. When tubed, the glycerin solution or water is added for maintaining the moisture.

**Potato Juice Media.**—**Potato Juice.**—White potatoes, grated (or run through chopping machine), 1 pound to 1 liter of water. Soak overnight. Heat to boiling. Press through cheese-cloth. Add one egg per liter. Autoclave one-half hour to clear. Filter through cotton (very tedious). Store in flasks and sterilize in autoclave one-half hour at 15 pounds' pressure.

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[1] Hyg. Rundschau, 1907, xvii, 1455.

**Agar.**—To veal agar add 5 per cent. potato juice and 5 per cent. glycerin.

**Broth.**—Meat infusion and potato juice are mixed in equal parts. Peptone and salt are added as in ordinary bouillon.

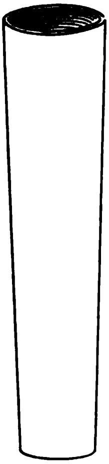

Fig. 43.—Potato borer.

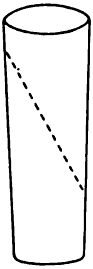

Fig. 44.—Cylinder of potato, line of cut.

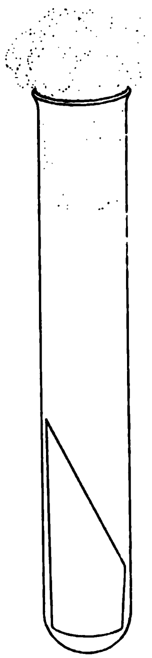

Fig. 45.—Finished medium.

**Bordet-Gengou.**[1]—Water 1000 c.c., glycerin 40 c.c., potatoes (sliced) 500 grams. Heat in an autoclave at 15 pounds' pressure for a half-hour. Pour off the liquid. To 500 c.c. of this potato extract add salt solution (0.6 per cent.) 1500 c.c., and agar 60 grams. Autoclave to dissolve, filter and tube. When used, an equal quantity or less of defibrinated blood is added.

**Synthetic Media.**—For certain work it is an advantage to know the exact chemical constitution of the medium. Then, too, some bacteria, especially certain species in soil, refuse to grow on the more complex media. Pigment production is very easily observed on these media.

**Uschinsky's** (Fränkel's Modification).[2]—Water 1000 c.c., asparagin 4 grams, ammonium lactate 6 grams, $Na_2HPO_4$ 2 grams, NaCl 5 grams.

**Modified Formula for Disinfection Tests.**—Water 1000 c.c., asparagin 6 grams, sodium phosphate (ortho) 2 grams, sodium chloride 5 grams. Dissolve and if necessary render alkaline to litmus by addition of NaOH. Sterilize in small tubes and test for color production with *B. pyocyaneus*.

**Ringer's Solution.**—Sodium chloride 10 grams, potassium chloride 0.2 gram, calcium chloride 0.2 gram, sodium bicarbonate 0.1 gram, glucose 1 gram, water 1000 c.c.

For broth add 1 or 2 per cent. peptone.

For agar add 1.5 or 2 per cent. agar and 1 or 2 per cent. peptone.

Dissolve, clear, and filter.

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[1] Ann. de l'Inst. Pasteur, 1906, xx, 731, (footnote, p. 734.)

[2] Hyg. Rundschau, 1894, iv, 769.

**Locke's Solution.**—To isotonic salt solution (0.9 to 1.0 sodium chloride) add 0.01 per cent. chloride of potassium and 0.02 per cent. chloride of calcium. This gives an "indifferent" solution, that is, one in which tissues neither swell nor shrink; in this respect it resembles the body fluids in that it prevents osmosis.

**Special Additions to Media.—Carbohydrates.**—These are added to media for two purposes: (1) to determine the presence of a *fermentative action* of the inoculated organisms with consequent production of acid and gas; (2) for *enrichment*, that is, as an added foodstuff to cause a more abundant growth. Carbohydrates may be used in any medium, either fluid or solid. When meat extract or meat infusion is used as a basis it must be remembered that both contain fermentable muscle sugar. If such a medium is prepared to determine whether a certain sugar is acted upon or not the muscle sugar must first be removed. This is done as follows:

**Preparation of Sugar-free Media.**—Adjust the reaction of the meat extract or infusion to about $P_h7$ (or slightly alkaline to litmus). To each liter add a broth culture of B. coli or one of its allies. Incubate for forty-eight hours. The bacillus ferments the sugar present. The infusion is then sterilized in the autoclave. Test again for production of acid or gas before using as a basic medium for addition of various carbohydrates. Serum water (Hiss, p. 122) may be used also as a basis.

Add carbohydrates to sugar-free media (see above), using only the purest carbohydrates obtainable. The following is a partial list of substances which may be employed. These are added usually in 1 per cent. amounts with the exception of glycerin; of this 5 per cent. is often used.

*Mono-hexoses*—dextrose, levulose, galactose, and mannose.

*Bi-hexoses*—saccharose, maltose, and lactose.

*Tri-hexoses*—raffinose.

*Polyhydric-alcohols*—erythrite (tetra), adonite (penta), and mannite, sorbite, dulcite, and glycerin (hexa).

*Pentoses*—arabinose, xylose, and rhamnose.

*Polysaccharids*—glycogen, dextrin, and inulin.

Besides the true carbohydrates, various glucosides, such as salicin, coniferin, etc., are used, as well as carbohydrate-like substances, like inosite.

Many of the carbohydrates are very susceptible to heat. They are split into simpler compounds which may be fermented by an organism unable to ferment the unaltered sugar and thus lead to error. The usual method is to add sufficient sugar to sterile water to make a 10 per cent. or 20 per cent. solution and heat this in small containers in the Arnold sterilizer. This solution is then added to the media in sufficient amounts to give a final 1 per cent. content.

Certain sugars, especially some samples of maltose, are so easily split by heat that they must be sterilized by filtration.

In routine work with glucose, lactose, saccharose, mannite, and dulcite it is usually sufficient to add the sugar to the media and sterilize by intermittent sterilization. Although there may be a slight destruction of the glucose, it is not enough to be important. Inulin is an exception. Because of the resistant spores commonly present, the inulin solution should be sterilized in the autoclave.

Fluid-sugar media, with the exception of serum-water media, are usually filled into fermentation tubes (p. 91), when gas production is to be determined.

**Addition of Enriching Substances.**—Serum, blood, albuminous body fluids, or tissue extracts may be added to media to give additional substances for producing growth.[1] This use of carbohydrates has already been given (see above).

Under aseptic conditions one part of the enriching substance is added usually to two or three parts of the medium. If additions are made to melted agar it must be cooled to 45° C.

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[1] See Rettger, L. F., for investigations concerning group factors, Jour. Bacteriol., 1918, iii, 103.

When slants are made, they should be allowed to set thoroughly, preferably overnight, before raising them, or they may slip down. For certain work it may be necessary to use a 2 per cent. agar to get sufficient stiffness so that the surface is not easily broken; this holds especially with media for plating where surface inoculation is done. Media thus prepared are spoken of as blood agar or broth, ascitic agar or broth, serum agar or broth, etc.

**Serum Media.**—For special purposes serum or other albuminous fluids, such as ascitic fluid, are used. They may be used as such or diluted with saline or by the addition of nutrient media. For these special purposes, sterilization by heat or even filtration should be avoided.

**Serum-water Media.**—If serum is dialyzed to remove the salts, it can be heated to 100° C. without coagulation. Serum diluted with two or three times its volume of distilled water can be sterilized in the Arnold sterilizer. This is so-called "serum-water medium" (Hiss[1]) to which 1 per cent. of sugar is added for fermentation tests. Acid production is shown by the change in the indicator added and by coagulation of the serum. Gas production is shown by the bubbles in the coagulum.

*Inulin serum-water* medium is the most commonly used preparation of this type. (See Carbohydrates.)

A similar medium is prepared as follows: Inulin (or other carbohydrate) 4 grams, peptone 4 grams, water, 200 c.c. Add litmus or other indicator. Dissolve and tube (2 or 3 c.c. to a tube) and sterilize, the method depending on the sugar used and precautions already given. After cooling add to each tube an equal quantity of sterile ascitic fluid or serum.

**Coagulated Serum for Production of Solid Media.**—Beef blood may be obtained at the slaughter house where it is placed in tall cylindrical vessels which are sterile. These should not be disturbed until the coagulation is complete. If the coagulum adheres to the sides of the vessel, it should be loosened with a sterile glass rod. After twenty-four hours on ice the serum is pipetted or syphoned off. If bloody, it can be placed on ice to allow the corpuscles to settle out. Dissolved hemoglobin is not desirable.

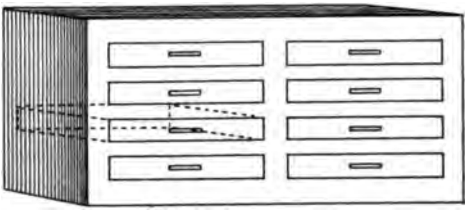

FIG. 46.—Serum inspissator (Park). Ringstands are used for support, and the tilt is easily varied as needed.

LÖFFLER'S BLOOD SERUM.—Mix 3 parts of beef or sheep serum and 1 part of nutrient broth (neutral to litmus, about $P_h$ 6.8 to 7) to which 1 per cent. of dextrose has been added. The mixture is run into tubes and the tubes slanted in an apparatus where the temperature can be slowly raised to between 80° and 90° C. The heating in any case should not exceed 95° C. until the medium is coagulated, or bubbling will occur and the surface will be spoiled. A Koch serum coagulator may be used for this purpose or a small water-oven or even an Arnold sterilizer, covering the top with a cloth instead of the usual cover. A convenient inspissator we have devised is shown (Fig. 46). It has the advantage that the temperature can be controlled and each tube heated equally. After coagulation the tubes should be sterilized in the Arnold sterilizer for twenty minutes on three successive days. The medium may be coagulated and sterilized in the autoclave by allowing the temperature to rise very slowly until 110° C. is reached. This is rapid and convenient, but it has seemed to us that the high temperature injured the medium somewhat.

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[1] Jour. Exper. Med., 1905, vi, 317.

COAGULATED BLOOD-SERUM MEDIA.—Serum from the cow, horse, sheep, or dog may be coagulated in the form of slants. This type of medium has been used with and without the addition of glycerin for cultivation of tubercle bacilli, but a more satisfactory medium can be prepared from eggs (p. 118). The serum used should be sterile and, after tubing, heated to 70° C. for one hour. If a clear medium is desired, the best way is to remove a tube from time to time for observation and cease heating when set sufficiently. Higher temperatures or too long an exposure render serum opaque.

Serum may be coagulated in high columns in test-tubes especially for cultivation of spirochetes. The tubes should be warmed to 65° C. and a few at a time placed in water at 75° C. These are tilted every few seconds and removed as soon as they start to set. The heat retained in the tube will complete the coagulation. In this way a soft, almost transparent, coagulum is formed.

The serum may be diluted with saline broth, etc., for special purposes and treated in the same way.

**Blood Media** (Hemoglobin).—Blood may be added to media in the form of whole blood, defibrinated blood or citrated blood. It must be obtained under aseptic conditions, as it cannot be sterilized. From the larger animals it is most easily obtained by introducing a sterile trocar into the jugular vein after carbolizing the site. The carbolic solution must be washed off with alcohol, which should be allowed to dry. With smaller animals anesthesia is used and the carotid artery is dissected out for the introduction of a trocar or cannula. Small quantities of blood may be obtained by puncturing the heart with a sterile syringe.

The fluid blood may be mixed at once with the medium. If the blood is to be stored it is either defibrinated by shaking it in the container, which contains sterile glass beads or spiral pieces of wire, until the fibrin has formed, or it is mixed with sodium citrate solution. One part of 10 per cent. sodium citrate solution is added to nine parts of blood.

Blood may be added to either fluid or solid media in the proportions given on page 121. Such media are opaque. Blood heated at a higher temperature than 45° C. makes better culture media for certain organisms. This is done by adding the blood to agar at 95° C., which produces the so-called **chocolate agar.** A final dilution of 1 to 500 gives a good growth with the influenza bacillus, though usually a dilution of 1 to 30 is used.

**Blood-drop Agar.**—A drop of blood may be added to the surface of solid agar, forming "blood-drop agar." This may be in the form of slants or plates. When these are inoculated the blood is streaked over the surface, together with the culture, by means of the platinum loop.

The chocolate and blood-drop agar are especially good for the influenza bacillus. The streptococcus and its allies grow well in all blood media.

**Tissues in Media.**—Watery extracts of tissues, especially of placenta, are used in the same manner as sera. Add 500 grams of ground-up tissue to 1 liter of water and extract as for meat infusion but apply no heat. After a preliminary filtration through paper or sand, the fluid is sterilized by filtration through a filter candle (p. 113).

**Use of Fresh Tissue in Media.**—Sterile fresh tissue may be added to media. This not only adds nutrient material but aids the production of anaërobic conditions. Smith[1] first used it in fermentation tubes for growing anaërobes, Williams[2] used it on agar for the growth of pure cultures of amebæ, and Noguchi[3] uses it in special media for spirochetes and other feebly growing organisms. In the latter medium rabbit's kidney is most commonly employed. Small pieces are added to fluid media or placed in test-tubes and serum or ascitic agar added. In the case of coagulated serum the tissue is pushed to the bottom of the tube.

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[1] Jour. Med. Res., 1905, xiv, 196.

[2] Ibid., 1911, xx, 263.

[3] Jour. Exp. Med., 1912, xvi, 199.

**Media Heated after the Addition of Tissues, etc.**—Especially good for the pathogenic (proteolytic) anaërobes. Drop a piece of meat into gelatin or broth tubes and sterilize; or cook the meat in water, alkalinize, sterilize and tube, *e. g.*, bullock's heart minced finely and then ground in a mortar, 8 ounces to tap-water 8 ounces.

Egg protein may also be used. Add one egg to 300 c.c. of water, mix, and bring to boil slowly, shaking frequently, tube and sterilize. An alkaline-egg mixture, yolk of one egg and whites of two, to 500 c.c. of water and 6 c.c. of N/1 NaOH may be used (as under Cholera Media) in proportion of 1 to 5 in broth or agar. (Robertson.)[1] See also milk, and inspissated serum or egg media for other specially suitable media.

**Potato Juice** (p. 119).—Potato juice may be added either alone or with glycerin. To veal agar add 5 per cent. potato juice and 5 per cent. glycerin. This is **glycerin potato agar.**

**Potato Broth.**— Meat infusion and potato juice are mixed in equal parts. Peptone and salt as in ordinary broth.

**Nutrose.**—May be used in media in 1 per cent. amounts to aid growth. The difficulty of obtaining nutrose during the war led R. L. McKenzie Wallis[2] to investigate its real composition and to suggest the use of a substitute nutrose prepared as follows: Peanut flour 94 parts, casein 5 parts, and sodium carbonate 1 part are mixed to form a powder. This is used as the powdered nutrose. The peanut flour and casein are insoluble in water unless the sodium carbonate is present.

This substitute is apparently helpful, but judging from some experiments made with the Conradi-Drigalsky medium prepared without any nutrose at all, it would seem to be not really necessary.

**Retention of Special Substances.**—**Vitamin Agar.**—In this agar special nutritive substances, "vitamins" are retained by the process of preparation. Lloyd[3] has shown that in the contact of media with cotton and paper, for the purpose of filtration, the vitamins are removed. This may be prevented by the use of sedimentation instead of filtration or by the use of the Sharples centrifuge.

To 500 grams of fresh beef heart, trimmed free from fat and tendon, add 500 c.c. of tap water, peptone 15 grams, sodium chloride 5 grams and 1 egg well beaten. Heat this mixture (A) in a water-bath, double boiler or over the open flame, with constant stirring, until the color changes to brown (at about 68° to 70° C.). Strain through a fine wire sieve or wire gauze. Do not use cheesecloth, cotton or paper.

Dissolve 15 grams of agar in 500 c.c. of tap water, mixture (B). Cool this mixture to 70° C. and add to (A). Set the reaction either to +0.2 phenolphthalein or to about $P_h7.4$. Heat the mixture in an autoclave at 15 pounds' pressure for thirty minutes. Remove the kettle carefully and set aside for sedimentation (p. 108) to take place, or run in Sharples centrifuge. If the sedimentation method is used, turn the solid agar out of the kettle in mould form and trim off the layer of sediment and discard it. The remainder is fairly clear.

Melt, tube and sterilize in an autoclave, at fifteen pounds, for thirty minutes.

**Use of Insoluble Carbonates.**—The value of the carbonates in media lies in the fact that they neutralize the acids produced by bacterial growth. These acids, produced especially when sugars are present, tend to prevent further growth or even injure the viability of the culture. The exact manner in which the carbonates act is not known. They are added chiefly to fluid media either in the form of calcium carbonate powder or of crushed marble. About 1 per cent. by bulk is sufficient. The marble must be white, not streaked with pink or brown. It is broken into pieces the size of a pea or smaller, washed in running water and sterilized in the dry air sterilizer before being added to the medium. Sterilize the medium in the autoclave at 15 pounds' pressure on two successive days as resistant spores are often present.

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[1] Jour. Path. and Bact., 1916, xx, 327.

[2] Indian Jour. Med. Res., 1917, iv, 4, 786.

[3] Jour. Path. and Bacteriol., 1916, xxi, 113.

Broth prepared with these additions is known as *carbonate* or *calcium* broth. It is useful especially for the streptococcus-pneumococcus group. When the medium is used in large amounts the containers should be shaken from time to time.

**Addition of Inhibiting Substances.—Anilin Dyes.**—Various basic anilin dyes show a differential restraining action on the growth of bacteria. Gentian violet (and allied dyes) when present in a dilution of 1 to 100,000 preferably in agar, inhibits the growth of the Gram-positive group of bacteria but has no effect on the growth of the Gram-negative bacteria. This is a general rule. It may be made use of to prevent growth of Gram-positive germs or for isolation of Gram-negative germs from contaminated material where the contamination is Gram-positive. An example of this is the use of crystal violet in Conradi medium. Exceptions to the above general rule occur, thus the acid-fast but Gram-positive group grow freely even when gentian violet in strong concentrations is used. Because of this, pure cultures of acid-fast bacilli (B. tuberculosis) can be obtained by this means from contaminated material, as sputum or feces.

**Gentian-violet-egg Medium** (Petroff).[1]—Meat extract (veal or beef 500 grams to 500 c.c. of 15 per cent. glycerin in water, extract for twenty-four hours and collect fluid by means of a press; sterilize by filtration). Add 1 c.c. of a 1 per cent. alcoholic solution of gentian violet to each 100 c.c. of meat extract, mix with 2 parts of whole egg (p. 118), tube and inspissate on three days for three-quarters of an hour at 80° to 85° C. This medium is especially recommended for the isolation of B. tuberculosis.

**Special Media for Typhoid, Paratyphoid, Dysentery, and Colon.**—Certain dyes have a differential action on closely allied bacteria inhibiting completely or partially the growth of one, but allowing another to grow freely. An example of this is the action of brilliant green on the typhoid, paratyphoid, and colon bacillus. The colon types are completely or nearly completely restrained, typhoid less so, and paratyphoid least of all. This is applied in isolation of typhoid or paratyphoid from stools. (See below.) The use of dyes as indicators has been already noted (p. 106). Numerous media are used. The most commonly employed plating media are Endo and Conradi-Drigalsky.

**Endo Medium (Kendall's Modification).**[2]—The basis is ordinary beef extract agar, slightly alkaline to litmus or about $P_h7$. This should be sterilized in bottles in 100 c.c. amounts. When needed, 1 gram of lactose is poured into a bottle of agar and the agar melted, which dissolves and sterilizes the lactose. To each bottle, after melting, is added 1 c.c. of decolorized fuchsin prepared as follows: To 10 c.c. of a freshly prepared 10 per cent. watery solution of sodium sulphite add 1 c.c. of a saturated alcoholic solution of fuchsin and heat in the Arnold sterilizer for twenty minutes. Plates are then poured and allowed to harden without the covers, and dried in the incubator for thirty minutes, protecting the plates from dust. The medium must be mixed each time it is needed and the plates used fresh for the color returns gradually and the plates are then useless. (See also Media for Water Examinations.) Kligler and Defandorfer recommend a reaction of $P_h7.6$ to 7.8 for the basic agar, the substitution of the bisulphite salt and the addition of 0.5 c.c. of the fuchsin sulphite mixture, for the B. dysenteriæ group.

**Robinson and Rettger's Modification of Endo's Medium.**[3]—This medium has given sharper differentiation than the above medium.

Preparation of agar: Water, 1000 c.c.; agar, 25 grams; peptone (Fairchild's), 10 grams; meat extract, 5 grams. Dissolve the agar, meat extract, and peptone. Make neutral to litmus (about $P_h6.8$), autoclave for thirty minutes at 15 pounds' pressure. Filter through cotton and cheese-cloth. Add 10 c.c. of a 10 per cent. sodium carbonate solution; heat for ten minutes; add 1 per cent. lactose,

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[1] Jour. Exper. Med., 1915, xxi, 38.

[2] Jour. Med. Res., 1911, xxv, 95.

[3] Ibid., 1916, xxix, 363.

5 c.c. saturated alcoholic fuchsin, and finally 10 c.c. of a 10 per cent. solution of anhydrous sodium bisulphite. The medium is now tubed in 20 c.c. amounts and autoclaved for seven minutes at 10 pounds' pressure. The best results are obtained when the reaction of the medium is brought, after the addition of the lactose and sodium bisulphite, before adding the fuchsin, to +0.1 phenolphthalein, hot titration.

According to our experience it seems better to prepare the medium, adjust the reaction to −0.4 (about $P_h$8.5). The sterilization and the subsequent addition of the bisulphite brings the reaction to the desired end-point; bottle in 100 c.c. amounts, and add the lactose, fuchsin, and sodium bisulphite just before use.

**Methylene Blue-eosin Agar** (Holt-Harris and Teague).[1]—Basis, extract agar of reaction of +0.8. Add lactose and saccharose, 0.5 per cent. and then to each 50 c.c. of agar, 1 c.c. of a 2 per cent. yellowish eosin solution and 1 c.c. of a 0.5 per cent. methylene blue solution. Shake and pour plates. The agar acts as a protective colloid preventing the mutual precipitation of the eosin and methylene blue. This protection is destroyed when acid is produced in the colonies, which then show as black or show black centers. If the plates are too crowded this change may not develop. The saccharose is omitted when used for dysentery isolation. B dysenteriæ (Shiga) may fail to grow on this medium.

**Conradi-Drigalsky Medium.**[2]—Water 1000 c.c., agar 20 grams, sodium chloride 5 grams, peptone 20 grams, nutrose 10 grams, beef extract (Liebig's) 4 grams, normal NaOH 50 c.c. Dissolve the ingredients in an autoclave, cool, and clear with eggs. Adjust reaction to a moderate but distinct alkalinity to litmus. To each liter of agar thus prepared are added 130 c.c. of Kubel and Thiemann litmus solution, crystal violet (1 to 1000 solution) 10 c.c., and 15 grams of lactose. Heat in an Arnold sterilizer ten minutes to obtain thorough mixing and fill in tubes or bottles and sterilize in Arnold sterilizer. Omit the crystal violet if to be used for dysentery.

**Russell's Double Sugar Medium.**[3]—To ordinary beet extract agar adjusted neutral to litmus add 1 per cent. of lactose and 0.1 per cent. of glucose and sufficient litmus to give a good color. Or add 1 per cent. of Andrade indicator in place of litmus and adjust reaction to this indicator. Tube and slant, leaving a generous "butt" at bottom of tube for stab inoculation. We have found it of great help to add 1 per cent. of saccharose, when using the medium in paratyphoid isolation. The fermentation of saccharose excludes the paratyphoid-like "intermediates" frequently found in stools.

**Brilliant Green Agar (Krumwiede) for Isolation of B. Typhosus and B. Paratyphosus.**—The basis of the medium is prepared as follows:

A. Water 1 liter, agar 30 grams; autoclave into solution.

B. Water 1 liter, meat extract 6 grams, salt 10 grams, peptone 20 grams; Arnold until dissolved.

Mix A and B; add normal soda (see below); boil one-half hour. Cool, clear with egg, filter until clear, bottle and autoclave. The agar, when used, must be neutral to Andrade's indicator (p. 106); it is convenient to adjust it at the time of preparation. For trial, 8 c.c. of soda per liter are added before clearing, and the agar finished. At the time of use, the reaction is tested, and if not found satisfactory, adjusted; allowance is made in subsequent batches so that, with the same brand of peptone, it is possible to obtain a constant and uniform reaction without titration. We find it convenient, on account of the necessity of standardizing the agar, to prepare large batches (20 liters); if this is done, the entire bulk of agar intended for use in brilliant green medium must be well mixed in one container after filtering. At the time the medium is to be used, a number of bottles are melted, and to each 100 c.c. is added 1 c.c. of indicator (Andrade's), 5 c.c. of a sterile solution in distilled water of 20 per cent. of lactose and 2 per cent. of glucose (this will give 1 per cent. lactose and 0.1 per cent.

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[1] Jour. Inf. Dis., June 6, 1916, xviii,

[2] Ztschr. f. Hyg., 1902, xxxix, 283.

[3] Jour. Med. Res., 1911, xx, 217.

glucose in the agar), and finally the appropriate amount (see below) of a 0.1 per cent. solution of brilliant green in distilled water. The medium is well mixed and poured into rather thick plates (no more than six from 100 c.c.). Porous tops are convenient, as dry plates are essential.

The selective bactericidal action of brilliant green is exhibited only at certain high dilutions. It is quantitative and varies according to the medium and the material inoculated. Even with similarly prepared batches of agar, different amounts of dye may be required to obtain the same restraining action. It is therefore necessary to determine for each batch the optimal dilutions for use, and these dilutions and the same dye solution are used for the rest of that preparation.

The method of standardization is to pour plates of four dilutions of dye: 1 to 500,000, 1 to 330,000, 1 to 250,000, 1 to 200,000, which correspond to 0.2, 0.3, 0.4, 0.5 c.c. of 0.1 per cent. dye solution to 100 c.c. of agar. These plates are evenly inoculated with broth cultures of freshly isolated strains of B. typhosus which have been diluted to give 75 to 200 organisms per loopful (loop of broth culture to 15 c.c. of broth), and with dilutions of broth cultures in suspensions of normal stools. This gives fairly satisfactory results. The best material for standardization is positive typhoid stools. A plate of each dilution of dye and a control plate of a non-restraining medium, such as Endo's, are used for each culture or stool.

The appropriate dilutions are (1) the lowest dilution at which the typhoid colonies are of good size and undiminished in number as compared with the control plate, but many fecal types are excluded; (2) a lower dilution, where the typhoid colonies are slightly reduced in size and number, but almost all the other flora have disappeared, the degree varying with different stools. They correspond, in general, to the effect we obtain with 0.2 and 0.3 c.c. respectively. For paratyphoid, the dye can usually be safely used in lower dilutions: 1 to 200,000. In investigating an outbreak of paratyphoid the medium can be standardized directly against the offending strain as soon as one is isolated. We have found two dilutions preferable for routine examinations, as the fecal proteins, which vary in amount in different suspensions, reduce somewhat the activity of the dye; and, moreover, the fecal flora of different stools as well as various strains of typhoid vary in their dye sensitiveness. With two concentrations these variables are balanced. For an occasional examination with unstandardized materials, 0.2, 0.3, 0.4 (0.5 if paratyphoid is suspected) will probably cover the range of variation.

To inoculate, the stool is emulsified (if fluid, it may be slightly diluted) in about 15 volumes of saline or peptone solution and the heavier particles are allowed to sediment for one-half hour. For routine work, two strong dye, two weak dye and two Endo plates are used for each sample; one loopful of suspension is placed on a weak dye plate, the same on a strong plate; these are evenly smeared with a heavy platinum or nichrome spreader, which is then carried over to an Endo plate which has received no direct material; a second series of weak and strong dye plates is inoculated with twice this amount, and spread in the same way to an Endo plate. The spatulum is not flamed between plates. This will usually give satisfactory seeding of Endo as well as dye plates, although the former receive only the material carried over on the spreader. If stronger dye is used, as for paratyphoid, more material may be inoculated.

After eighteen hours' incubation the typhoid colonies are characteristic in appearance. They are of good size (1 or 1.5 mm.), as the glucose favors their growth. Viewed through the plate against a dark background, with the light passing obliquely through the agar, they have a peculiar marking; with artificial light and hand lens, under the same conditions, they have the appearance of a coarse wool fabric. The colony may have a slight pinkish tinge from fermentation of the trace of glucose; this renders it still more distinctive. The larger colonies resemble paratyphoid bacillus; they are heavier, and more opaque, the markings are less evident, and they may be tinted a delicate green. Paratyphoid A resembles the typhoid colony more nearly than the paratyphoid B; it is frequently extremely flattened, slightly tinged, and has an ill-defined edge. All the types show better markings on stronger dye. On weak dye plates (rarely on strong) there may develop typhoid-like colonies of coliform organisms, but their exaggerated striations, with distinct crossbars, usually exclude them. Many of these typhoid-like organisms agglutinate spontaneously on the slide when the macroscopic slide technic is used for identification (see Agglutination).[1]

Digest Products for Basic Agar.—Amino-acid digests are more favorable for the growth of members of this group than are the extract-agars or even infusion media. Cole and Onslow have shown this by the acceleration of early carbohydrate fermentation in such media. Stickel and Meyer recommend especially a peptic liver digest basis (see p. 117).

**Fluid Media Containing Brilliant Green.**—Browning, Gilmour and Mackie,[2] Krumwiede and Pratt,[3] Torrey,[4] Robinson and Rettger[5] and many English workers have successfully used brilliant green in peptone-water or broth, sometimes with the addition of glucose, for the isolation of typhoid or paratyphoid bacilli from feces. It is essential to use graded dilutions of the dye; dilutions from 1 to 100,000 to 1 to 500,000 are generally advised. The medium is sterilized in 5 or 10 c.c. quantities in test-tubes, and the dye added just before use; After twelve to eighteen hours the highest concentration tubes showing growth are plated on Endo's medium. While the pathogenic organisms are frequently lost in this medium by overgrowth of mucoid aërogenes types, enrichment in brilliant green peptone-water or broth may give positive results where even brilliant green agar fails; for routine work, the above fluid media add too much to the time, cost and labor of examination to warrant their use except in special instances. Teague and Clurman[6] have recently reported a medium which they claim gives better results.

**Special Media for Cholera.**—The vibrio group will grow in the presence of a reaction so extremely alkaline that there is inhibition of the growth of most other bacteria, especially those which occur in feces.

**Peptone Solution** (see p. 98).

**Saccharose Peptone-water** (Bendick).[7]—To 1000 c.c. peptone solution, neutralized to phenolphthalein add 1 gram of anhydrous sodium carbonate. Boil and filter. Add 5 grams of saccharose and 5 c.c. of a saturated solution of phenolphthalein in 50 per cent. alcohol. Tube and sterilize in Arnold sterilizer.

**Dieudonne's Medium.**[8]—Mix equal parts of defibrinated beef blood and normal sodium or potassium hydrate solution and steam in the Arnold sterilizer for a half-hour; 3 parts of this are added to 7 parts of 3 per cent. agar neutral to litmus, (about $P_h6.8$) and poured into Petri dishes (15 c.c. to a 10 cm. dish). Let them harden uncovered, but protected by paper. Place strips of filter paper between the dish and cover to aid in the absorption of the moisture and ammonia and place in the incubator for twelve to fifteen hours. Nothing will grow on the medium when first made. The plates are good for about ten to fourteen days. Various modifications of this medium have been suggested.

Pilon.—Substitute 12 per cent. sodium carbonate (crystalline) solution for the sodium hydrate. The plates can be used after thirty minutes' drying.

**Alkaline-egg Medium** (Krumwiede).—Make an egg-water mixture, using equal parts of egg and water. Mix equal parts of the egg-water and 12 per cent. sodium carbonate (crystalline) and steam in the Arnold sterilizer for twenty minutes. While hot mix 30 parts to 70 parts of 3 per cent. beef extract agar, reaction unadjusted, pour into plates and dry for twenty minutes. The colonies are increased in size if 0.5 per cent. of glucose or saccharose is added. Goldberger[9] claims that the alkalinity is unnecessarily high, and makes the egg mixture, as given, with a 6.5 per cent. solution of anhydrous sodium carbonate and mixes 1 part of the egg with 5 parts of glucose extract agar.

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[1] Jour. Infect. Dis., 1918, xxiii, 275.

[2] Jour. Hyg., 1913, xiii, 335.

[3] Jour. Exper. Med., 1914, xix, 20 and 501.

[4] Jour. Infect. Dis., 1913, xiii, 263.

[5] Jour. Med. Res., 1911, xxix, 363.

[6] Ibid., 1916, xxxv, 107.

[7] Centralbl. Orig., 1st Abt., 1912, lxii, 536.

[8] Centralbl. f. Bakteriol., Orig., 1909, l, 107.

[9] Hyg. Lab. Bull., December, 1913, No. 91, p. 19,

**Sodium Oleate Agar** (Avery).—This is a selective medium for the isolation of the influenza bacillus. By the action of the sodium oleate the growth of many of the Gram-positive cocci, present in nasal secretions or in the sputum, is inhibited when these materials are inoculated on this medium. It is prepared as follows:

1. A 2 per cent. nutrient agar (vitamin, p. 124, preferred) either neutral in reaction (*i. e.*, about $P_h7$) or slightly alkaline ($P_h7.2$ or 7.4). If phenolphthalein is used as the indicator make the end-reaction somewhat acid to it, that is, about +0.4.
2. Sodium oleate, neutral (Kahlbaum's preferred), a 2 per cent. stock solution in water. This may be sterilized in the autoclave.
3. Suspension of red-blood corpuscles. Sterile defibrinated human or rabbit blood may be used. Remove the corpuscles by centrifugation and discard the supernatant serum by pipetting it off. Make up the original volume by the addition of nutrient broth. Mixture: To 94 c.c. of the hot agar (90° C.) add 5 c.c. of the 2 per cent. sodium oleate solution and 1 c.c. of the red-blood cell suspension.

**Media for Special Uses other than Those Given.**—The media for the detection of the typhoid-dysentery-colon group (p. 125), for the diphtheria bacillus (Chap. XX), for the tubercle bacillus (pp. 119 and 123), for the influenza bacillus (pp. 123 and above), the pneumococcus and the streptococcus (pp. 123 and 125) have been given.

**Liver Agar** (recommended by Dopter, Penfold and others).—Substitute minced liver for meat and proceed as in preparing infusion agar. With a reaction of +0.2 per cent. to phenolphthalein it is especially adapted for gonococcus.

**Starch Medium.**—(Vedder.)—Beef 500 grams, water 1000 c.c., extract in ice-chest overnight, boil and strain, add agar 1.5 per cent. and dissolve. Correct reaction to +0.2 to +0.7 per cent. phenolphthalein, *i. e.*, between $P_h7$ and $P_h7.6$. Cool and clarify with eggs. Filter, add 1 per cent. of starch and heat in Arnold for forty-five minutes, shaking the medium several times to distribute the starch. Tube and autoclave fifteen minutes at 10 pounds' pressure. Recommended especially for meningococcus.

**Media for the Examination of Milk, Water,[1] Shell-fish and Sewage.**—*Standard Methods.*—The basis for broth, agar or gelatin is 3 grams of beef extract (Liebig's or its equivalent by tests) and 5 grams of peptone (Witte's or its equivalent) to 1 liter of distilled water. No salt is used. Gelatin media contain 10 grams of gelatin dried at 105° C. for one-half hour before weighing. Agar media, 1.2 per cent. agar if dried as above, or 1.5 per cent. if market agar is used. The agar should be soaked in water and washed after weighing to rid it of its salts. If the reaction of the medium does not fall between +0.5 and +1 (phenolphthalein) or about $P_h6.6$ to $P_h7.4$, correct it (see Reaction, p. 100). Usually correction is not necessary, especially with agar.

Sugar broths are prepared by the addition of 1 per cent. of the required carbohydrate. The reaction of sugar broth should be neutral to phenolphthalein, about $P_h8$. Litmus (reagent of highest purity) should be in 2 per cent. solution. Azolitmin (Kahlbaum), 1 per cent. solution, may be used as a substitute. Adjustment of reaction of the solution may be necessary. One cubic centimeter of litmus solution is added to 10 c.c. of lactose agar for litmus-lactose-agar plates when poured. All media and litmus solutions are to be tubed before sterilization and sterilized at 15 pounds' pressure for fifteen minutes, removed quickly and cooled rapidly.

The Endo recommended has as a basis an agar containing 3 per cent. of agar, 1 per cent. of peptone and 0.5 per cent. of Liebig's extract. The medium is sterilized in 100 c.c. lots. Just before use add to the melted agar 1 gram of lactose and 0.5 c.c. of a mixture of 10 c.c. of a 10 per cent. solution of sodium sulphite and 2 c.c. of a 10 per cent. solution of basic fuchsin in 95 per cent. alcohol. (See Media for Typhoid.) For presumptive tests for B. coli, lactose broth in fermentation tubes is to be used.

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[1] Committee on Standard Methods of Water Analysis, 1917. A. P. H. A., 93.

**Milk.**—Agar for milk work is prepared as follows:

*Procedure A.*—Dissolve the beef extract, 0.3 per cent., and peptone,[1] 0.5 per cent. in two-fifths of the total amount of distilled water by boiling on the stove. Filter this mixture through paper or paper pulp (p. 108).

*Procedure B.*—Agar, oven-dried, 1.2 per cent., or market agar, 1.5 per cent. Soak and wash under tap in sieve; add it to distilled water, three-fifths total amount of water minus the water absorbed by the agar during the washing. Determine this by first weighing kettle with unwashed agar and then with washed agar which has been well drained. The weight of 1 gram is considered as 1 c.c. of water absorbed.

Mix A and B (agar not yet melted). Heat mixture over stove, stirring at frequent intervals until agar is entirely melted. Then boil and stir constantly for twenty minutes; make up water lost in evaporation by adding hot distilled water. Do this by weight.

Determine the reaction (see above). Do not use eggs for clearing. Filter through cotton or paper-pulp in Buchner funnel (p. 108) or run through a Sharples centrifuge until clear. Take 10 c.c. to a tube for plates. Sterilize in autoclave for twenty minutes after the pressure reaches fifteen pounds, or in streaming steam on three successive days for twenty minutes each day after agar is completely melted.

**Water.**—The preparation of agar for water work differs[2] slightly from that given for milk. That the milk agar be adopted for both is to be recommended by the Committee.

**Shell-fish.**—Use agar as for water count. Lactose bile (see below) is still employed.

**Additional Media for Water, Sewage, Shell-fish Examinations.**—**Neutral Red Lactose Peptone.**—To peptone solution add 1 per cent. of lactose and 1 per cent. of a saturated aqueous solution of neutral red. Tube in fermentation tubes.

**Liver Broth.**—Beef liver 500 grams, peptone 10 grams, dextrose 10 grams, dipotassium phosphate ($K_2HPO_4$) 1 gram, water 1000 c.c. The liver is boiled for two hours for extraction and strained. Then dissolve other ingredients and adjust reaction.

**Lactose Bile.**—Add 1 per cent. of peptone and 1 per cent. of lactose to ox bile. Tube in fermentation tubes and sterilize.

**Media for Toxin Production, etc.**—**Diphtheria Toxin Broth** (Smith).—Lean veal (chopped), 20 pounds; water (tap), 20 liters. Place in ice-box overnight; next morning, strain. Titrate at room temperature and set reaction 1 per cent. acid to phenolphthalein. When chill is removed from kettle, add 10 c.c. of twenty-four hour culture B. coli for every liter and incubate at 37° C. overnight. Next morning add 1 egg for every 2 liters. Boil for twenty minutes and strain. Titrate as before and set reaction 0.5 acid (phenolphthalein). Then add: peptone,[3] 1 per cent., NaCl, 0.5 per cent. Heat to boiling to dissolve peptone and salt. Titrate and make final reaction 0.5 per cent. acid to phenolphthalein. Filter through filter paper and cotton. Put 800 c.c. in each 2-liter Erlenmeyer flask. Sterilize in autoclave at 15 pounds for one-half hour. Before inoculating flasks add to each one 8 c.c. of a 10 per cent. solution of glucose which has been sterilized in Arnold on three successive days for thirty minutes.

**Diphtheria Toxin Broth** (Banzhaf).—Lean veal free from fibrous tissues, minced, 1 pound to the liter of water. Soak overnight and then heat to between 45° and 55° C. for two hours. Then bring to a boil. Strain. To fluid add peptone (Witte) 1.5 per cent. and sodium chloride 0.5 per cent. Boil for a half-hour.

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[1] If it is found by experience that the peptone used requires the addition of normal NaOH to give the required reaction, add a suitable amount at this point. Do not add acid under any circumstances.

[2] After the agar and other ingredients are dissolved the cooling to 45° C. in a cold water bath and then warming to 55° C. in the same bath without stirring is directed.

[3] Witte, Berna or Eimer and Amend.

Adjust reaction to 1.2 per cent. acid to phenolphthalein or about $P_h$7. Place about 800 c.c. in 2-liter Erlenmeyer flasks and add 3 grams of calcium carbonate. Sterilize in an autoclave at fifteen pounds' pressure for a half hour.

**Diphtheria Toxin Broth** (Martin's Peptone). — Lean veal (market) free from fibrous tissue, minced, one pound to the liter of water. Incubate for eighteen to twenty-four hours at 35° C. Heat at 45° to 48° C. for one hour. Boil briskly for a half hour. Strain through cheese-cloth. Measure the filtrate and warm it to 70° C. Add an equal amount of Martin's peptone (p. 116) which has just been neutralized (about $P_h$ 7) at 70° C. A clear product usually results from mixing the two at this temperature. Heat to boiling. Titrate (room-temperature method) to +0.5 (phenolphthalein). Autoclave at about ten pounds' pressure for fifteen to twenty minutes to clear. Titrate again and adjust to +0.5 as before if necessary. Filter through cotton and paper into flasks, allowing 800 c.c. to a flask. Sterilize one-half hour at fifteen pounds' pressure.

**Tetanus Toxin Broth.** — One pound of lean veal per liter of water. Soak overnight in ice-chest. Heat to 45° C. for one hour, then boil for one-half hour. Strain, add peptone (Witte) 1 per cent., salt 0.5 per cent. and glucose 1 per cent. Boil until ingredients are melted and adjust reaction to +1 per cent. Sterilize in 2-liter Erlenmeyer flasks, in Arnold sterilizer, leaving only sufficient space in flask for expansion during heating. Heat for one and a half hours on first day and for one hour on second day.

**Tetanus Toxin Broth** (Martin's Peptone). — Lean veal (market) free from fibrinous tissue, minced, 1 pound to the liter of water. Soak in ice-box for eighteen to twenty-four hours. Heat to 45° to 48° C. for one hour. Boil briskly for one-half hour. Strain through cheese-cloth. Measure the filtrate and warm to 70° C. Add an equal amount of Martin's peptone which has just been neutralized at 70° C. The two liquids should be at the same temperature, for then a clear product usually results. Heat to boiling. Titrate (room-temperature method) to +1 (phenolphthalein). Autoclave at about ten pounds' pressure to clear. Measure and add 1 per cent. glucose (powder). Boil two or three minutes. Titrate again and set reaction as before to +1. Filter through cotton and paper directly into 2-liter Erlenmeyer flasks, leaving only sufficient space in flasks for expansion of broth during sterilization in the Arnold. Sterilize flasks for one and a half hours on the first day and one hour on second day.

**Mallein Broth** (Eye and Subcutaneous).—Lean veal free from fascia, minced, one pound to the liter of water. Macerate overnight at room temperature. Heat at 45° to 50° C. for one hour. Boil up strongly. Strain through cheesecloth. Add peptone (Witte) 1 per cent., sodium chloride 0.5 per cent. Boil again to dissolve the peptone. Titrate (room-temperature method) and adjust reaction to +2.5 (phenolphthalein) (about $P_h$ 6 to $P_h$ 6.4). Autoclave for fifteen minutes at fifteen pounds' pressure to clear. Filter through cotton and paper. Measure and add glycerin, 5 per cent. Put 250 c.c. broth in each quart Blake bottle or liter flask. Sterilize in autoclave one-half hour at fifteen pounds' pressure.

**Media for Protozoa.—For Blood Flagellates** (Novy and MacNeal[1]).—Equal parts of nutrient agar and fresh defibrinated blood (rabbit or rat). The medium is allowed to stiffen slanted, so more water of condensation may gather at bottom of slanted surface. The medium should be planted while fresh with blood or other infected material containing the living flagellates (*e. g.*, trypanosomes, Leishmania).

**Medium for Malaria Organisms** (Bass and Johns[2]).—10 c.c. of blood drawn from a malarial patient, and carefully defibrinated. This is placed in smaller test-tubes in 1 c.c. amounts. One per cent. of a 50 per cent. solution of dextrose is added to each small test-tube before adding the blood. The red corpuscles settle so that a ½ cm. layer of serum is left about them. The parasites grow in a thin layer near the top of the cell sediments. Beneath this zone the parasites die. Bass says leukocytes should be gotten rid of because they destroy the parasites, but the Thompsons say this is unnecessary. Most favorable incubation temperature 39° to 40° C.

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[1] Contribution to Med. Res., dedicated to V. C. Vaughan, Ann Arbor, 1903, p. 549; also Jour. Infect. Dis., 1904, i, 1.

[2] Jour. Exper. Med., 1912, xvi, 567.

**Ameba Agar** (Musgrave and Clegg[1]).—Agar 1 per cent., tap-water 90 per cent., ordinary nutrient broth 10 per cent. Mix and sterilize as usual. Reaction about neutral to phenolphthalein.

**Medium for Pure Cultures of Amebæ** (Williams).[2]—Fresh sterile brain, liver or kidney is cut in small pieces and placed on the surface of ameba agar.

**Special Media for Yeasts and Moulds.**—Most of the pathogenic yeasts and moulds can be cultivated on the media already described. Growth is much improved if glucose or maltose is added. The reaction should, however, be acid or at most slightly alkaline to litmus.

For moulds, especially those infecting the skin, an agar containing no meat but 1 to 2 per cent. peptone and 2 per cent. of glucose or maltose and 0.5 per cent. of glycerin with no change of reaction (*Sabouraud*[3]) gives a very favorable medium.

For yeasts, beerwort media are especially favorable. Hopped beerwort is obtained at a brewery. Autoclave, cool and filter and then tube (this avoids subsequent precipitation). For solid media add 10 per cent. gelatin or 1.5 per cent. agar. Various carbohydrates may be added in 2 per cent. amounts.

## CULTIVATION OF MICROÖRGANISMS.

Microörganisms can seldom be identified by the microscope alone. By this method certain characteristics, such as individual form, arrangement, individual reaction to stain, and motility or lack of motility can be studied. But in order to learn all the characteristics of an organism, including its specific relation to disease, we must be able to grow it in pure culture, that is, apart from all other organisms, and determine its cultural characteristics on various media, its immunity reactions and its action in the animal body. Koch's postulates (see p. 22) were based on the ability to obtain pure cultures. In cultivating microörganisms it is not only necessary to supply the appropriate foodstuff, but also to have the appropriate conditions as to temperature, moisture, access of oxygen, etc.

When we make cultures of any material, we are likely to find that instead of only one variety of microörganism, there are a number present. If we grow the material in *fluid media*, we find that as the different varieties develop they spread through the medium and become hopelessly mixed; furthermore, the more vigorous varieties outgrow the feebler ones, which are thus lost. If, however, the microörganisms in the material be scattered through or on a *solid medium*, they may grow in separate colonies which may be visible to the naked eye or can be seen with a low-power lens. From each of these colonies a pure culture may be obtained.

**Methods of Isolating Pure Cultures.**—*Plating Methods.*—Two plating methods are employed: pour plates or streak plates. Agar or gelatin media are used. The medium is first rendered fluid: gelatin, by placing the tubes in warm water (35° to 40° C.); agar, by immersing the tubes in boiling water until fluid and then cooling the tubes to between 40° and 42° C. Agar melts completely only when heated to the boiling-point of water or thereabouts and hardens as the temperature falls below 40° C. The cooling of the agar is necessary, otherwise the organisms would be injured by the heat. If enriching fluids are to be used, they are added to the agar when between 45° and 50° C. The material from which we wish to obtain pure cultures is then inoculated into the fluidified media. The method of inoculation (see Methods of Inoculating Culture Media, p. 138) is in general as follows: A loopful of the material is carried into a tube of the medium and thoroughly mixed with the medium by tilting and rolling the tube. Agitation sufficient to produce bubbles must be avoided. From this tube three loops are carried over to a second tube which is mixed in the same manner. From the second tube five loops are carried to a third tube and mixed. The lips of the tubes are flamed and the contents of each poured into a Petri dish, which is, if necessary, tilted to spread the agar evenly over the bottom of the dish. In pouring, the lid of the dish is raised just sufficiently to pour the contents of the tube into the dish, the lid acting as a protection against falling dust. In diluting the material with culture media, it is our object to separate the organisms so that when colonies develop from them they will be well separated, that is, *discrete*. If they are not well separated, we should touch several colonies in attempting to transfer the growth of one and thus fail to obtain pure cultures. As we have no way of telling what dilution will give us well-separated colonies, a series of dilutions is necessary. The method will have to vary with the probable number of organisms in the material. If they are in great numbers, further dilutions of the material will be necessary, as the first tube will give overcrowded plates. A preliminary dilution or two may be made in saline solution or broth. When the material contains very few organisms, more material must be inoculated into the first tube. The dilutions may be made with a sterile pipette instead of a loop. The tip of the pipette is touched to the material and carried over to the first tube of medium, mixing being done by sucking the medium up and down in the pipette; about 0.1 c.c. of the contents of this tube is carried to a second tube, 0.1 c.c. of this to a third, etc.

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[1] Manila Bureau of Public Printing, 1904.

[2] Jour. Med. Res., 1911, xx, 263.

[3] Ann. de dermat. et de syph., 1892 and 1893.

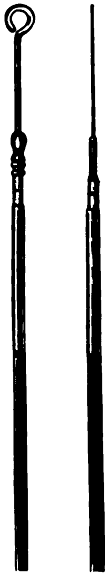

FIG. 47. — Platinum needle and loop. For most purposes finer wire is used.

*Streak Plates*.—In this method the medium (agar) is poured into Petri dishes and allowed to harden. The material is then streaked over the surface of the medium either with a platinum loop or spatula or a sterile bent-glass rod. Two or more plates are inoculated in succession without further sterilization of the loop or spatula. This dilutes the material so that discrete colonies are obtained. A difficulty that is likely to arise in the use of streak plates, and to a less degree with pour plates, is collection of water of condensation, which, finding its way to the surface of the plate, will cause a spreading of the growth over the whole surface. This can be avoided to a large extent by cooling the agar before pouring it in the plates. Special covers made of porous earthenware can be obtained, which will absorb the moisture. Incubating the plates upside down is a good precaution. Motile bacteria are most likely to spread over the surface of media. The special media used for the isolation of fecal bacteria must, because of the great numbers of motile bacteria in the stool, be comparatively dry. As most of these media restrain the ordinary contaminating bacteria from growing, they may be allowed to harden and dry in the Petri dishes with the covers off, and the drying continued by placing them in the incubator partly open.

*Fishing Colonies.*—This is the term used for the process of transferring the microörganisms from the colony to a fresh medium. This is usually done with a straight platinum wire, which is less likely to touch any colony other than the one we wish to fish. Fishing is done either by selecting the colony with the unaided eye or by the aid of a microscope. The advantage gained by the use of the microscope is that we can be sure that the colony to be fished has no microscopic colonies in the immediate neighborhood likely to be touched by the platinum wire. Furthermore, if the fishing is done under the microscope, the fact that one and only one colony has been touched is visually proved. The colony to be fished is centered under a No. 2 or AA objective and the wire introduced between the objective and the agar until the tip of the wire touches the colony. The progress of the wire is watched through the microscope to be sure that nothing is touched but the colony, and to observe by the broken appearance of the colony that it has been touched. The sense of touch is relied on to tell whether the objective is encountered. Opaque media are fished by eye. The plate is held against the light and the colony touched with the wire. In either case the organism is transferred to a fresh medium—usually an agar slant—by rubbing the tip of the wire over the surface of the medium.

*Possible Sources of Error in Plating Methods.*—Theoretically, each colony is the progeny of one single organism, or in the case of organisms that adhere to form pairs, chains, etc., the progeny of such an aggregate of one variety of organism. Practically it happens not infrequently that dissimilar varieties cling together and a colony develops containing both. A second plating from such a colony is usually sufficient to yield pure cultures. In exceptional instances there is the greatest difficulty in separating such "clingers" and it is sometimes impossible.

**Special Methods of Isolation.**—*Dilution Method.*—It is conceivable by gradual dilution of material to reach a point at which some of the loops of the material may only contain one organism which when carried to tubes of medium will yield pure cultures. This method was employed before the introduction of solid media. The uncertainty of the method is evident. For certain purposes modified dilutions are still employed, starting usually with cultures obtained from colonies on plates, the method being employed not so much to obtain pure cultures as to obtain cultures known to be the progeny of one cell.

*Hansen's Method for Yeasts.*—Cover-slips are given a thin coating of paraffin, through which lines are drawn dividing the cover into small squares and each numbered. The marking is done with a needle. The covers are now exposed to hydrofluoric acid to etch the markings into the glass. They are then cleaned and sterilized. Dilute the yeast-containing material with fluidified gelatin until one loop averages one yeast cell as determined by microscopic examination. The gelatin is then spread over the surface of the sterile cover-slip, hardened and inverted over a Böttcher moist chamber, sealing with vaselin. This is placed under the microscope and the position of the single cells determined, making a sketch for later reference. Incubate at room temperature and when colonies have developed fish those developing from single cells. The advantage of this method over plating is that on plates a proportion of the colonies will develop from adhering cells. The purity of yeasts in industries is of enormous financial importance.

*India-ink Method* (Burri)[1].—The use of india-ink for microscopic examination has already been described. By making successive dilutions of small amounts of culture in drops of ink it is possible to make small spots of ink containing only one organism. This is determined microscopically. This is the starting-point for the pure culture from one cell. The reader is referred to the article by Burri for the technical details. The method has only a very limited application (see page 75).

*Capillary Method* (Barber[2] and others).—Reference is made to the original articles for the details of this method for isolating one cell.

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[1] Das Fuscheverfahren als einfaches Mittel, etc., Jena, G. Fisher, 1909.

[2] Kansas Univ. Sc. Bull., 1907, iv, 41; also Jour. Infect. Dis., 1908, v, 380.

*Preliminary Enrichment Methods.*—Very often it is impossible to isolate certain organisms from material because they are too few in number. With certain organisms it is possible to inoculate the material in special media which will allow free multiplication of this organism, but be unfavorable for the others contained in the material. Examples of this are the use of Löffler's medium for mixed cultures from the throat to find diphtheria, or the peptone method for the enrichment of cholera and other vibrios. Another method is the use of ascitic broth for the isolation of diphtheria from mixed material. The last two methods take advantage of the surface growth of the bacteria. The same end is attained by the use of the following factors: Blood media for hemoglobinophilic bacilli; increase of the alkalinity, as in selective media for vibrios; increased acidity, as with tartaric acid, for yeasts; dyestuffs, gentian violet to inhibit Gram-positive bacteria; brilliant green to inhibit the colon types in mixed cultures from stools; use of heat, spore-containing mixed cultures being heated to 80° C., to kill the non-sporebearing types; motility, the motile organism spreading from the point of inoculation of the mixed material, as with amebæ and spirochete-containing material; filtration through filters, spirochetes passing filters, most bacteria being held back (see Filtrable Viruses); germicidal agents (see Anilin Dyes, p. 125); antiformin for separation of acid-fasts from other bacteria (see Tuberculosis, Chap. XXVII); finally, the use of animals (same chapter).

*Special Methods for Anaërobes.*—(See Anaërobic Methods.)

**Applications of the Plating Methods.**—*Study of Colony.*—The appearance of the colony is one of the points used in identifying bacteria; or the colony appearance being known, the isolation of a certain organism is greatly simplified. In plates showing two or more types of colonies it is an aid in determining the relative proportion of the subsequently identified varieties in the material examined.

In examining colonies both the macroscopic and microscopic characteristics are determined. The age at which a colony will be most characteristic depends on the rapidity of growth of the organism. Daily observations are most suited for full information. As a rule young colonies are most characteristic, although such characteristics as pigment production and gelatin liquefaction may only appear on longer incubation. With the naked eye the following points should be observed: Moist or dry, transparent, translucent or opaque, edge sharply defined or indefinite, regular or irregular outline, fringe-like margin or thread-like outgrowths, color, flat, raised or umbilicated, etc. On microscopic examination the presence or absence of granules, whether fine or coarse, their arrangement and distribution, should be observed, as well as the finer details of the characteristics mentioned under the naked-eye examination. The observations are usually made with a low-power objective, No. 2 or No. 3. The fine details may be observed with a higher lens and the arrangement of the bacteria especially at the edge of the colony can be observed. The accompanying figures illustrate the colony characteristics mentioned.

*Determination of the Number of Bacteria.*—If a measured amount of fluid is mixed with a plating medium we should theoretically be able to determine the number of bacteria by counting the number of colonies that develop. This would be true if each bacterium was completely separate, if each was able to multiply, if the medium was satisfactory for the development of each variety contained in the material, and if the oxygen access, moisture and temperature of incubation were favorable for the growth of all the varieties. This is an impossibility. Most nearly accurate results are obtained where only one or at most several varieties are present and the conditions for their growth maintained. Although the method gives an incorrect result when mixed material is used, the enumeration of the colonies that develop is of a great deal of practical value, as in the examination of milk and water. If the methods employed are always the same, the comparison of the results gives us valuable information as to the bacterial content.

The method employed is in general as follows: To 9 c.c. of water is added

1 c.c. of the material to be examined; this is thoroughly shaken to separate the organisms; 1 c.c. of this is then added to 9 c.c. of water thoroughly shaken, and 1 c.c. of this added to 9 c.c. of water and so on. This gives a dilution of 1 to 10, 1 to 100, 1 to 1000, etc. How far to dilute the material must be determined from the character and probable bacterial content. One c.c. of each of these dilutions is placed in a Petri dish and the melted plating medium poured into the dish, the two being mixed by tilting the dish. Mixing can be done by adding the dilutions to the tubes of plating medium and pouring the content into the dishes; but as all the medium cannot be poured out of the tube, some bacteria are left behind. It is easier, however, to obtain even distribution of the colonies in this way, and for comparative work the method gives reliable results. The counts by this method average about 10 per cent. lower than where mixing is done in the dish.

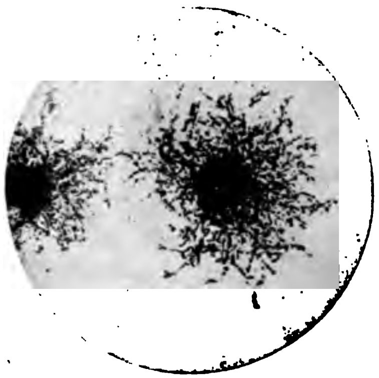

FIG. 48.—Irregular fringed colony (B. malignant edema). (From Kolle and Wassermann.)

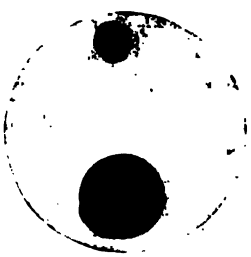

FIG. 49.—Colonies of typhoid and colon bacilli in rather soft gelatin.

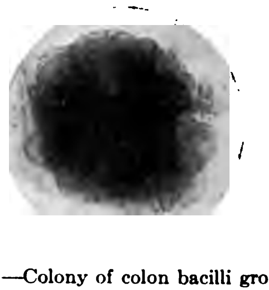

FIG. 50.—Colony of colon bacilli grown in soft gelatin.

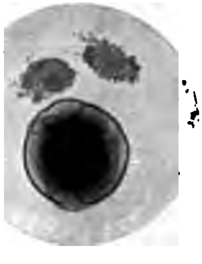

FIG. 51.—One large irregular colony of colon and two smaller colonies of typhoid bacilli in soft gelatin. (Figs. 49 to 51 from photographs by Dunham.)

In place of dilutions made by pipettes, a series of loops or rings on a platinum needle can be employed which hold a known quantity (small) of the material to be examined.

To obtain a satisfactory count the colonies should be about 100 to 200 to a plate. Lower numbers than this are too few to give a fair average, higher numbers are difficult to count because the colonies have not sufficient space for full development, some of the colonies become confluent, and some of the feebler bacteria are checked in their development by the crowding and do not develop colonies. The plate that shows about 100 colonies is chosen, therefore, for counting. When no one plate is completely satisfactory in number and even distribution of the colonies, two of the nearest satisfactory plates are counted and the results averaged. Whenever possible all the colonies on a plate should be counted. Where the colonies are crowded it is necessary to divide the plate into sections to facilitate counting. This is best done by placing the Petri dish on a Wolffhügel counting plate, which is a glass plate ruled in square centimeters, some of the squares being still further divided into 9 small squares. A row of squares is counted and then a row at right angles to the first, to obtain a fair average. The number of colonies is divided by the

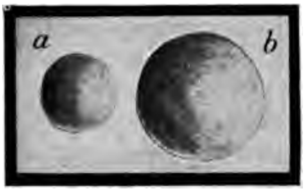

Fig. 52.—Moist raised colonies with no visible structure, looking like a drop of water. (Figs. 52-59 from Lehman and Neumann.)

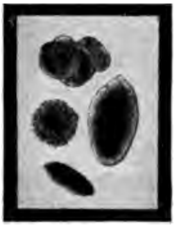

Fig. 53.—Deep colonies, usually either light brown, gray or yellow in color, opaque with little marking.

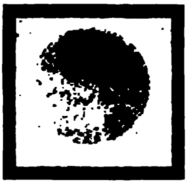

Fig. 54.—The colony very finely granular.

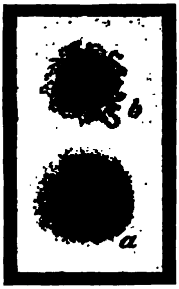

Fig. 55.—Colonies opaque in center with lighter borders. The margin is coarsely granular, or has twisted threads.

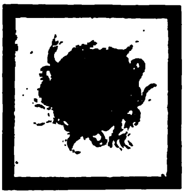

Fig. 56.—Colony in gelatin. The center is coarsely granular in partly fluid gelatin. The borders are formed of wavy bands of threads.

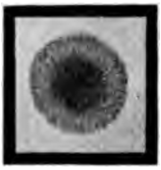

Fig. 57.—Colonies circular in form, composed of radiating threads.

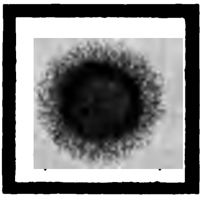

Fig. 58.—Colonies with opaque centers, with a thin border fringe.

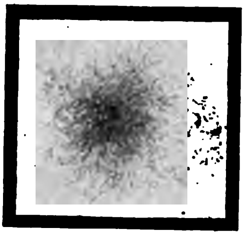

Fig. 59.—Colony showing a network of threads which is thicker in the center.

number of squares counted. If the usual 10 c.c. Petri dish is used, the number per square centimeter is multipled by 63, the number of square centimeters on the dish, to give the total number. This is multiplied by the dilution used in this dish, which gives the colonies developing from 1 c.c. of the material used for plating. If dishes of another size are used, the area in square centimeters is determined by multiplying the diameter by 3.1416.

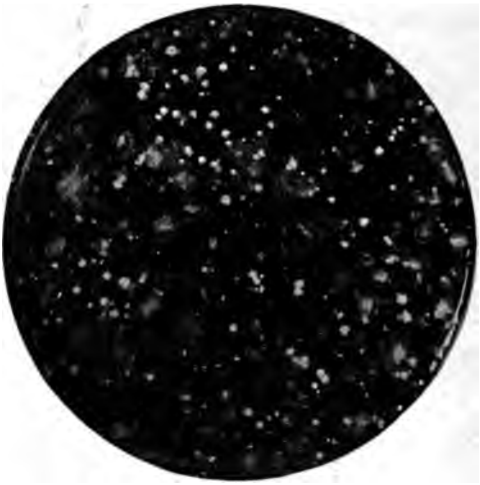

FIG. 60.—Photograph of a large number of colonies developing in a layer of gelatin contained in a small Petri dish. Some colonies are only pin-point in size; some as large as the end of a pencil. The colonies here appear in their actual size.

**Methods of Inoculating Culture Media.**—The inoculation of plating media has been described under the methods of isolating pure cultures. Platinum wire is usually employed for transferring the growth from one medium to another. The wire is either attached to the end of a glass rod or to special aluminum holders which are made for this purpose. Straight wire or wire with the end bent into a loop is used. The wire is sterilized by heating it in the flame of a Bunsen burner until red-hot. Care must be taken that the wire is cool before it is used. The straight wire is used for fishing colonies or where tubed solid media is to be inoculated by long puncture through the media (stab

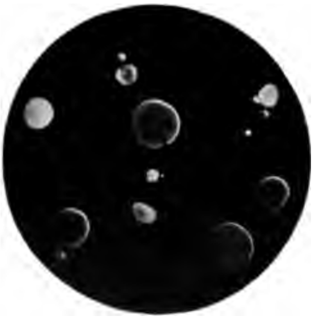

FIG. 61.—Well-distributed colonies in agar in portion of Petri dish.

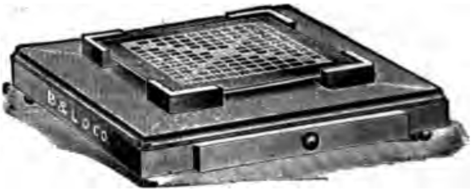

FIG. 62.—Wolffhügel's apparatus for counting colonies.

culture). The loop is used for ordinary transfers. In transferring from solid media the loop is drawn upward over the medium, filling the loop with the growth; this is rubbed over the surface of solid media or in the case of fluid media against the glass at upper level of the fluid. In transferring fluid cultures a loop of the fluid is used. When the growth on fluid media is in the form of a

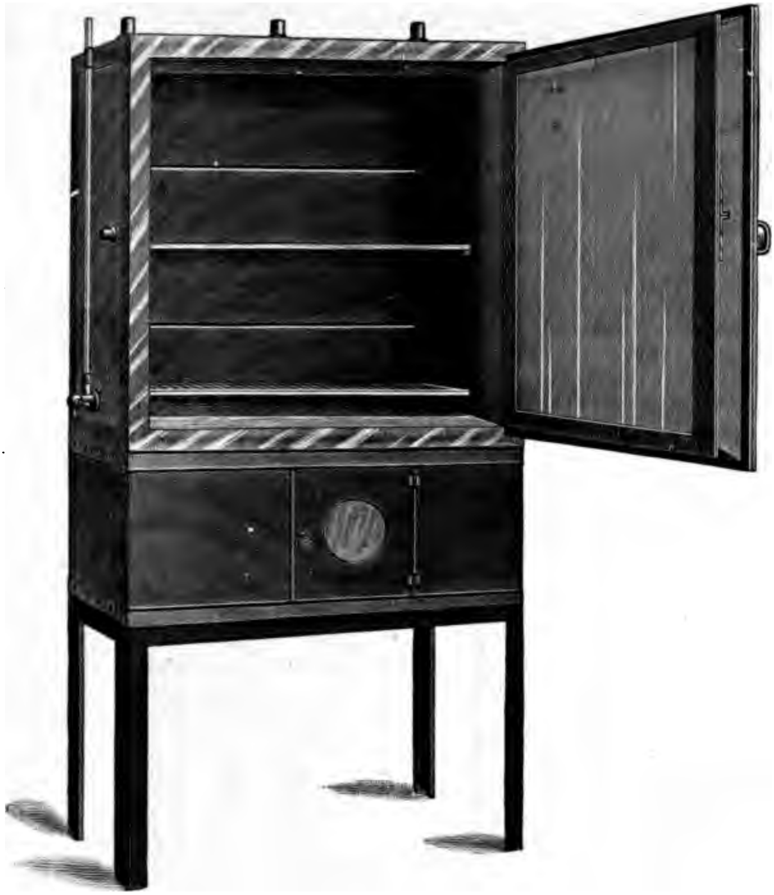

Fig. 63.—Incubator.

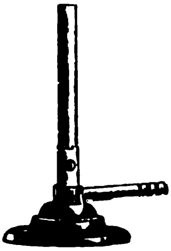

Fig. 64.—Bunsen burner, ordinary type.

pellicle it may be necessary to inoculate this pellicle so that it floats or growth may not take place. In transplanting, the tubes or other containers must not be kept open any longer than necessary or contamination is likely to occur. In transferring from and to tubed medium the tubes are held by placing them against the palm and fingers of the left hand, grasping the tubes by the butt between the thumb and the palm, low enough so that the contents of the tube are not covered by the thumb. The right hand holding the sterilized platinum wire is used to remove the cotton plugs, one being grasped by the small finger and the other between the fourth and fifth fingers. After drawing out the plugs the necks of the tubes are flamed and the growth then transferred and the plugs replaced; the platinum wire is then sterilized at once before laying it down to mark the tubes. The marking is done usually with a wax pencil made for writing on glass. The plugs should be protected from dust and just before use should be moistened with a weak antiseptic.

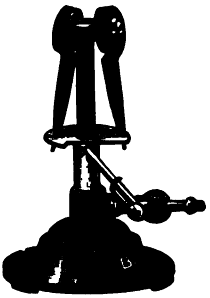

Fig. 65.—Safety-burner.

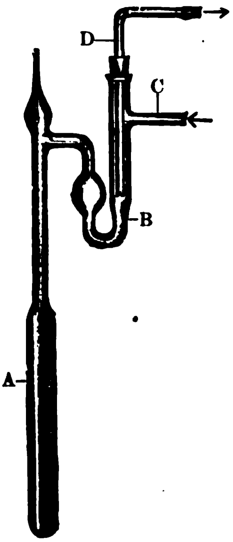

Fig. 66.—Heat regulator. (Dunham.)

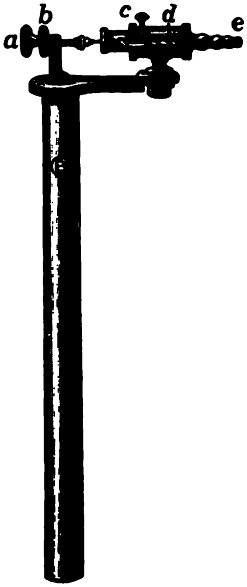

Fig. 67.—Heat regulator. (Roux.) Bimetallic.

**Apparatus for and Methods of Incubation.**—The selection of the temperature for incubation depends on the organism to be grown and the medium employed. Although the optimum temperature for growth may vary between 5° and 50° C. or more, the great majority of organisms will grow at either 22° or 37° C. These are the temperatures usually employed. For general purposes the former is obtained by incubation at room temperature. Where a more exact and uniform temperature is necessary an incubator must be employed. This is a double-walled oven, the space between the walls being filled with water; heat is supplied by gas or electricity and cooling by the addition of cold water. The latter is only necessary when the outside temperature rises above 22° C. The heating or the addition of water is controlled by an automatic regulator. To maintain a temperature of 37° C. a similar incubator is employed, the heating, however, being continuous; an automatic regulator controls the temperature. The heat-controlling devices are of varied construction, the expansion of a fluid or the change in shape of a bar of two unequally expanding metals is used to make and break the electric contact or open and close the gas vent. In the former mercury is used alone or at the point of control. When a large incubating room is required, a room can be heated with a large gas stove, a funnel being placed over the stove and connected with this a radiator-like arrangement of 4- to 6-inch pipes ending in a chimney-like outlet for the products of combustion. The radiator distributes the heat more evenly through the room. In spite of this there will be a variation in temperature in different parts of the room, the shelves near the radiator or nearest the ceiling being the warmer. This is an advantage rather than a disadvantage, as a range of about 5° is available for special purposes. In using gas, all connections are best made of metal; flexible metal tubing is available. Small burners are preferably of the safety type, which, if extinguished, turn off the gas automatically. In the case of gas stoves there should be an independent pilot flame not influenced by the regulator, which will relight the stove in case of accident.

**Oxygen Requirement Methods.**—Where aërobic conditions are required the access of air to the growth is all that is necessary. For anaërobic conditions the oxygen must be removed. This is done (1) by exhaustion of the air, (2) by chemical absorption of the oxygen, (3) by displacement of the air as by hydrogen, or (4) by growing in the depths of solid media, or in fluid media under a layer of albolene to prevent the reabsorption of air driven off during sterilization; (5) or, lastly, by a combination of these methods. The addition of fresh animal tissues to media for the enhancement of the growth of anaërobic organisms has been mentioned under Media. Occasionally reducing substances are added directly to the medium, or the organism is grown in symbiosis with an aërobic bacillus which absorbs the oxygen.

The group of organisms spoken of as "micro-aërophilic" will only grow when the oxygen is partly removed. This may be done (1) by exhaustion of the air to measured degree, (2) by growing the organism in a sealed jar containing also a stated number of cultures of an organism that absorbs oxygen in its growth, as B. subtilis, or (3) by mixing the material with melted agar in tubes and allowing the agar to set, the development taking place at the depth that has the favorable oxygen content. In the second method the optimum number of square centimeters of B. subtilis culture per liter of air must be known.

*Anaërobic Methods.*—For exhaustion a pump is required; the filter pumps for attachment to the faucet are the most convenient where water under pressure is available. Any type of pump, however, may be employed. The extent of exhaustion can be measured by a manometer. Any type of jar of appropriate dimensions with a tight-fitting cover and a stop-cock opening can be used. The Novy jar is a satisfactory type. Museum jars with a side opening are very satisfactory substitutes. The jar must be absolutely tight or the vacuum will not be held. Absorption can be added to exhaustion by placing pyrogallic acid and a stick of sodium hydrate in the bottom of the jar. After exhaustion is completed a small amount of water is allowed to be sucked back into the jar. This dissolves the sodium hydrate and the resulting reaction of the sodium hydrate with the pyrogallic acid is accompanied by absorption of the oxygen from the air remaining in the jar.

Hydrogen may be used to replace the exhausted air. After exhaustion, hydrogen from a Kipp apparatus is sucked back into the jar diluting the remaining air; the jar is again exhausted and then filled with hydrogen, again exhausted and filled. Only traces of air will remain, and if absolute anaërobiasis is required pyrogallic acid may be used to absorb this last trace as above, allowing the water to be sucked back before the negative pressure is completely relieved by the hydrogen. In relieving the negative pressure it may be necessary to use a pneumatic trough to collect the hydrogen, as the generation of hydrogen will be too slow and air will be drawn through the Kipp apparatus. It is advisable not to relieve the negative pressure completely, as this helps to hold the lid tightly in place, and furthermore, the subsequent expansion when the jar is incubated will tend to loosen the lid and allow inward diffusion of air. When the pyrogallic method is used, the tubes or plates must be raised above the level of the fluid. Of the methods given, the second and third are the most satisfactory. The absorption method may be used alone, using a jar to hold the tubes or plates; or individual plates or tubes may be handled as in the following:

*Buchner's Method.*[1]—The culture tube is placed in a larger tube, at the bottom of which is placed the pyrogallic acid, on which some sodium hydrate is poured and the tube quickly closed with a rubber stopper, or the NaOH may be put in dry and water added.

*Wright's Method.*[2]—The tubes are plugged with absorbent cotton; after inoculation of the medium the plug is pushed into the tube, pyrogallic placed on the stopper, sodium hydrate solution added, and the tube quickly corked.

*Zinsser's Method for Plates.*[3]—The dishes employed must be deeper than the ordinary Petri dishes. The agar is poured in the smaller dish in the ordinary manner, and as soon as hard inverted over moistened filter paper to protect it from contamination. In the larger dish is placed the pyrogallic acid and the smaller dish placed in it. One side of the smaller dish is raised and the hydrate solution poured in the space between the two dishes and the inner dish dropped back. As rapidly as possible albolene is run in the space between the dishes from a pipette previously filled so that no time is lost.

*The methods of exclusion of oxygen* are simple and can be, in most instances, substituted for the more cumbersome methods described. Plating may be done with the ordinary Petri dishes as follows (Krumwiede and Pratt[4]): The bottom part of the dish, open side up, is placed in the cover and sterilized in this shape and protected from dust until used. The plating medium is inoculated as though pour plates were to be

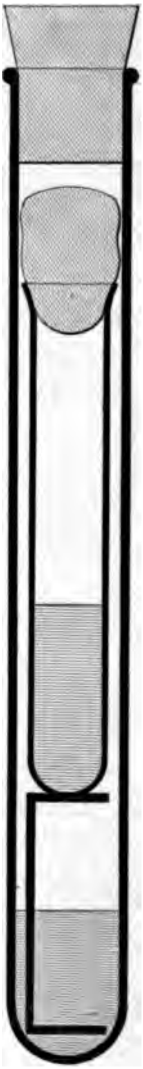

FIG. 69.—Buchner's anaërobic tube The fluid consists of pyrogallic acid dissolved in 10 per cent. NaOH solution. By Wilson's method the tubes are charged with pieces of caustic potash covered with pyrogallic acid.

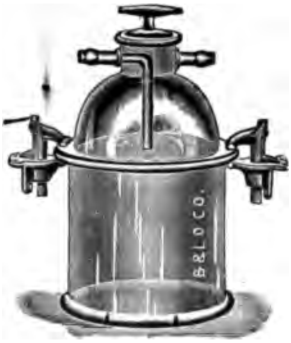

FIG. 68.—Novy jar for anaërobic cultures.

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[1] Centralbl. f. Bakteriol., 1888, 1st Abt., vol. iv.

[2] Jour. Boston Soc. Med. Sc., 1900, iv, 114.

[3] Jour. Exper. Med., 1906, viii, 542.

[4] Jour. Infect. Dis., 1913, xii, 199.

made in the ordinary way. The inoculated medium is then poured into the cover and the bottom of the dish laid on the fluid medium, tilting it so that the air can escape as it falls into place. With a little practice this can be done so that no bubbles are present (see Fig. 70). After the medium has set the edge is painted over with melted paraffin to prevent drying and contamination. If any of the medium flows over the edge, it is wiped away by carbolized cotton. After incubation the parts of the dish are carefully pulled apart and the colonies may be fished. As a rule the agar remains in the cover. If it should adhere to the other part of the dish, this can be laid in the cover to allow of fishing under the microscope. If gas-producing bacteria are present, fishing should be done as soon as sufficient growth has taken place or the gas will disrupt the medium and cause the growth to spread over the plate. If an actively motile organism is present, the method may fail because of spreading between the medium and the glass.

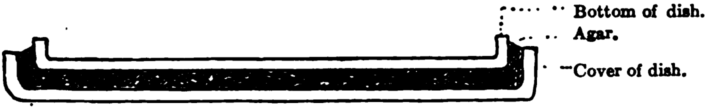

FIG. 70.—Anaërobic plating method.

Another method for the isolation of pure cultures is the *shake culture.* The material is diluted with a plating medium in the usual way, although more medium is used in the tubes. Instead of pouring the medium into plates the medium is allowed to solidify in the tubes. The colony desired can be obtained as follows: scratch the tube with a file at the level of the colony, then wet the tube with alcohol, burn this off and press on the scratch with a very hot piece of metal or glass rod. The tube will crack and the column of agar can be cut through to allow the two parts of the tube to be separated and the colony fished. This method has a limited application, but is especially useful when the organism to be isolated is a partial anaërobe and grows at a certain level in the agar. Pure cultures of these organisms may not be obtained at the first attempt, but the level of thickest growth may then be used, the first culture serving in this way as an enrichment.

For the cultivation of pure cultures the use of stab cultures in agar or gelatin may be employed. The most satisfactory medium for this purpose is a semisolid agar (Krumwiede and Pratt). Because of the softness of the medium the material for subinoculation is easily obtained; the stab inoculation closes better than if made in stiffer medium, and the medium does not dry out nor split so readily. For subinoculation a platinum wire bent like a corkscrew is a help in obtaining the material from the depths of the media. With spirochetes it is much easier to obtain the growth with the capillary pipette from about the tissue at the bottom of the tube than where the usual 1.5 per cent. agar is employed. The use of the capillary pipette for the transfer of cultures is open to much wider application with semisolid media, because if the medium has a satisfactory density, the ordinary rubber teat exerts sufficient suction to draw up the medium and a syringe is not needed.

The *exclusion of oxygen* from a fluid medium is most simply carried out by sterilizing the medium under a layer of albolene. This can be done either in tubes or flasks, preferably the globe flasks. In the latter the medium is filled to the neck and albolene added, leaving room for expansion; after sterilization and contraction of the medium more sterile albolene is added if necessary.

Occasionally anaërobes are grown in mixed culture with an aërobic organism to absorb the oxygen. One of the best organisms for this purpose is the hay bacillus.

As a general rule the addition of a fermentable carbohydrate to the medium gives much better growth of anaërobic organisms.

**The Study of Pure Cultures.**—The study of pure cultures is the b for classification and identification. The characteristics studied the morphology, staining reactions, and motility when grown on o both solid and fluid media (see Microscopic Study); the cultural cha teristics when grown on various media, the food, oxygen, and temp ture requirements, the reaction to the various immunity tests chapters on Immunity), and the action of the organism or its prod on experimental animals (see Use of Animals). The following short summary of the more important cultural reactions and the met of eliciting them.

**Cultural Characteristics.**—*Colony Morphology.*—(See Application of Pl Methods.)

**Growth on Agar and Other Solid Media** (*Surface Growth*).—Moist or dry, fl raised, flaky or easily emulsified, adherent to medium or not, mucoid or s smooth, irregular, or threaded margin, color or pigment production (see be extension into medium, etc.

**Growth in Solid Media.**— In stab culture, depth to which growth ext character of growth around the surface puncture, the spreading of the gr into the medium (an index of motility best shown in semisolid media), th like extensions from the line of puncture; in gelatin, liquefaction, non-l faction, rate, area and character if present; in shake cultures, the charac the colonies.

**Fluid Media.**—Pellicle, tenacious or easily broken, spreading up the si the tube or not, thick or thin, clear or cloudy, and degree of clouding, ment or deposit on sides of tube, granular, flaky, gelatinous, mucoid, or str stalactite formation, more densely clouded at one level than another.

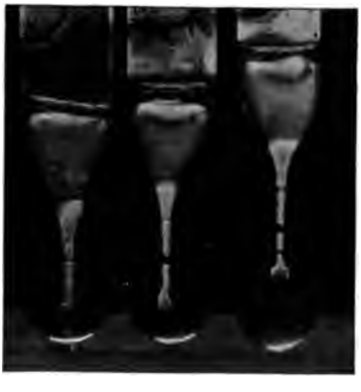

FIG. 71.—Stab cultures of three cholera spirilla in gelatin, showing in upper porti growth considerable liquefaction of nutrient gelatin.

**Food Requirements.**—Growth on simple or complex media, albumins su serum required, blood or hemoglobin necessary, carbohydrate needed for development (example—glycerin for tubercle bacillus).

**Oxygen Requirements.**—Growth only in free oxygen, obligate aërobe; gr only where oxygen is absent (depths of media, etc.), obligate anaërobe; gr under both conditions, facultative anaërobe (an available carbohydra necessary to elicit the facultative character); growth with a definite but less amount of oxygen (development only in one level of solid media), micro- philic. This last characteristic may be lost after cultivation for some tim artificial culture media.

**Source**.............................. **Date of Isolation**................ **N**

### DETAILED FEATURES.

NOTE—Underscore required terms. Observe notes and glossary of terms on opposite side of card.

### I. MORPHOLOGY (²)

1. **Vegetative Cells,** Medium used....................
temp..............age..............days
Form, *round, short rods, long rods, short chains, long chains, filaments, commas, short spirals, long spirals, clostridium, cuneate, clavate, curved.*
Limits of Size...............
Size of Majority............
Ends, *rounded, truncate, concave.*

Agar Hanging-block
Orientation (grouping)........
Chains (No. of elements)......
*Short chains, long chains*
Orientation of chains, *parallel, irregular.*

2. **Sporangia,** medium used..........temp...........
age..............days
Form, *elliptical, short rods, spindled, clavate, drumsticks.*
Limits of Size..........Size of Majority...........

Agar Hanging-block
Orientation (grouping)........
Chains (No. of elements)......
Orientation of chains, *parallel, irregular.*

Location of Endospores, *central, polar.*

3. **Endospores.**
Form, *round, elliptical, elongated.*
Limits of Size...............
Size of Majority............
Wall, *thick, thin.*
Sporangium wall, *adherent, not adherent.*
Germination, *equatorial, oblique, polar, bipolar, by stretching.*
4. **Flagella,** No.........Attachment *polar, bipolar, peritrichiate.* How Stained.........
5. **Capsules,** present on.............
6. **Zooglea, Pseudozooglea.**
7. **Involution Forms,** on........in.....days at....° C.
8. **Staining Reactions.**
1 : 10 watery fuchsin, gentian violet, carbol-fuchsin, Loeffler's alkaline methylene blue.
Special Stains,
Gram..................Glycogen...............
Fat....................Acid-fast...............
Neisser................

### II. CULTURAL FEATURES (³)

1. **Agar Stroke.**
Growth, *invisible, scanty, moderate, abundant.*
Form of growth, *filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.*
Elevation of growth, *flat, effuse, raised, convex.*
Lustre, *glistening, dull, cretaceous.*
Topography, *smooth, contoured, rugose, verrucose.*
Optical characters, *opaque, translucent, opalescent, iridescent.*
Chromogenesis (⁴)................
Odor, *absent, decided, resembling*............
Consistency, *slimy, butyrous, viscid, membranous, coriaceous, brittle.*
Medium *grayed, browned, reddened, blued, greened.*
2. **Potato.**
Growth *scanty, moderate, abundant, transient, persistent.*
Form of growth, *filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.*
Elevation of growth, *flat, effuse, raised, convex.*
Lustre, *glistening, dull, cretaceous.*
Topography, *smooth, contoured, rugose, verrucose.*
Chromogenesis (⁴)...........Pigment in water *insoluble, soluble:* other solvents.....................
Odor, *absent, decided, resembling*...................
Consistency, *slimy, butyrous, viscid, membranous, coriaceous, brittle.*
Medium, *grayed, browned, reddened, blued, greened.*
3. **Loeffler's Blood-serum.**
Stroke *invisible, scanty, moderate, abundant.*
Form of growth, *filiform, echinulate, beaded, spreading, plumose, arborescent, rhizoid.*
Elevation of growth, *flat, effuse, raised, convex.*
Lustre, *glistening, dull, cretaceous.*
Topography, *smooth, contoured, rugose, verrucose.*
Chromogenesis (⁴)...............
Medium *grayed, browned, reddened, blued, greened.*
Liquefaction begins in......d, complete in......d,
4. **Agar Stab.**
Growth *uniform, best at top, best at bottom:* surface growth *scanty, abundant: restricted, wide-spread.*
Line of puncture, *filiform, beaded, papillate, villous, plumose, arborescent: liquefaction.*
5. **Gelatin Stab.**
Growth *uniform, best at top, best at bottom*
Line of puncture, *filiform, beaded, pap*
*plumose, arborescent.*
Liquefaction *crateriform, napiform, inf*
*saccate, stratiform:* begins in.........
complete in...................d
Medium *fluorescent, browned*...........
6. **Nutrient Broth.**
Surface growth, *ring, pellicle, flocculent, none.*
Clouding *slight, moderate, strong: transier*
*none: fluid turbid.*
Odor, *absent, decided, resembling*.......
Sediment, *compact, flocculent, granular, on agitation, abundant, scant.*
7. **Milk.**
Clearing without coagulation.
Coagulation *prompt, delayed, absent.*
Extrusion of whey begins in............
Coagulum *slowly peptonized, rapidly pepto*
Peptonisation begins on....d, complete o
Reaction, 1d...., 2d...., 4d...., 10d..
Consistency, *slimy, viscid, unchanged.*
Medium *browned, reddened, blued, greened.*
Lab ferment, *present, absent.*
8. **Litmus Milk.**
*Acid, alkaline, acid then alkaline, no change*
*Prompt reduction, no reduction, partial al*
9. **Gelatin Colonies.**
Growth *slow, rapid.*
Form, *punctiform, round, irregular, amebo*
*filamentous, rhizoid.*
Elevation, *flat, effuse, raised, convex, pulvi*
*form* (*liquefying*).
Edge, *entire, undulate, lobate, erose, lacera*
*filamentous, floccose, curled.*
Liquefaction, *cup, saucer, spreading.*
10. **Agar Colonies.**
Growth *slow, rapid* (temperature........
Form, *punctiform, round, irregular, amebo*
*filamentous, rhizoid.*
Surface *smooth, rough, concentrically rin*
*striate.*
Elevation, *flat, effuse, raised, convex, pu*
*bonate.*
Edge, *entire, undulate, lobate, erose, lacera*
*floccose, curled.*
Internal structure, *amorphous, finely, coars*
*grumose, filamentous, floccose, curled.*
11. **Starch Jelly.**
Growth, *scanty, copious.*
Diastatic action, *absent, feeble, profound.*
Medium stained..................
12. **Silicate Jelly (Fermi's Solution).**
Growth *copious, scanty, absent.*
Medium stained.................
13. **Cohn's Solution.**
Growth *copious, scanty, absent.*
Medium *fluorescent, non-fluorescent.*
14. **Uschinsky's Solution.**
Growth *copious, scanty, absent.*
Fluid *viscid, not viscid.*
15. **Sodium Chloride in Bouillon.**
Per cent. inhibiting growth.............
16. **Growth in Bouillon over Chloroform,**
*feeble, absent.*
17. **Nitrogen.** Obtained from *peptone, aspa*
*coli, urea, ammonia salts, nitrogen.*
18. **Best media for long-continued growth**....
....................................
19. **Quick tests for differential purposes**.....
....................................
....................................

### III. PHYSICAL AND BIOCHEMICAL FE

| 1. Fermentation-tubes containing peptone-water or sugar-free bouillon and | Dextrose | Saccharose | Lactose | Maltose | Glycerin |
|---|---|---|---|---|---|
| Gas production, in per cent. | | | | | |
| $\left(\frac{H}{CO_2}\right)$ | | | | | |
| Growth in closed arm | | | | | |
| Amount of acid produced 1d. | | | | | |
| Amount of acid produced 2d. | | | | | |
| Amount of acid produced 3d. | | | | | |

ame.................................................. **Group No.** (1)..........................

i.
'llate, rillous,
undulatiform,
..........d.
...
membranous,
it, persistent:
...........
flaky, viscid

lays.
ised.
n....d.
.., 20d....

'.
no reduction.

'd, mycelioid,
nate, crateri-
te, fimbriate,

......)
d, mycelioid,
ged, radiate,
lcinate, um-
te, fimbriate,
ely granular,

...........
unrestrained,
agin, glyco-
...........
...........
...........
...........

:ATURES.

Mannit

2. **Ammonia production,** *feeble, moderate, strong, absent, masked by acids.*

3. **Nitrates in nitrate broth.**
*Reduced, not reduced.*
Presence of nitrites.......ammonia................
Presence of nitrates.......free nitrogen............

4. **Indol production,** *feeble, moderate, strong.*

5. **Toleration of Acids,** *great, medium, slight.*
*Acids tested*..............

6. **Toleration of NaOH,** *great, medium, slight.*

7. **Optimum reaction for growth** in bouillon, stated in terms of Fuller's scale..........................

8. **Vitality on culture media,** *brief, moderate, long.*

9. **Temperature relations.**
Thermal death-point (10) minutes' exposure in nutrient broth when this is adapted to growth of organism)............C.
Optimum temperature for growth......° C.; or best growth at 15° C., 20° C., 25° C., 30° C., 37° C., 40° C., 50° C., 60° C.
Maximum temperature for growth.......... ° C.
Minimum temperature for growth.......... ° C.

10. **Killed readily by drying:** resistant to drying.

11. **Per cent. killed by freezing** (salt and crushed ice or liquid air)................

12. **Sunlight:** Exposure on ice in thinly sown agar plates; one-half plate covered (time 15 minutes), *sensitive, not sensitive.*
Per cent. killed................

13. **Acids produced**................

14. **Alkalies produced**..............

15. **Alcohols**......................

16. **Ferments,** *pepsin, trypsin, diastase, invertase, pectase, cytase, tyrosinase, oxidase, peroxidase, lipase, catalase, glucase, galactase, lab, etc*..............................
..............................................

17. **Crystals formed:**.......................................

18. **Effect of germicides:**

| Substance | Method used | Minutes | Temperature | Killing quantity | Amt. required to restrain growth |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
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| | | | | | |
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| | | | | | |
| | | | | | |

## IV. PATHOGENICITY.

1. **Pathogenic to Animals.**
*Insects, crustaceans, fishes, reptiles, birds, mice, rats, guinea-pigs, rabbits, dogs, cats, sheep, goats, cattle, horses, monkeys, man*.............................

2. **Pathogenic to Plants:**
..............................................
..............................................
..............................................

3. **Toxins,** *soluble, endotoxins.*

4. **Non-toxin forming.**

5. **Immunity bactericidal.**

6. **Immunity non-bactericidal.**

7. **Loss of virulence on culture-media:** *prompt, gradual, not observed in*..........................months.

**BRIEF CHARACTERIZATI**

Mark + or O, and when two occur on a line erase the one does not apply unless both ap

| | | |
|---|---|---|
| MORPHOLOGY (2) | | Diameter over 1μ |
| | | Chains, filaments |
| | | Endospores |
| | | Capsules |
| | | Zooglea, Pseudozooglea |
| | | Motile |
| | | Involution forms |
| CULTURAL FEATURES (3) | | Gram's stain |
| | Broth | Cloudy, turbid |
| | | Ring |
| | | Pellicle |
| | | Sediment |
| | Agar | Shining |
| | | Dull |
| | | Wrinkled |
| | | Chromogenic |
| | Gel. plate | Round |
| | | Proteus-like |
| | | Rhizoid |
| | | Filamentous |
| | | Curled |
| | Gel. Stab. | Surface growth |
| | | Needle growth |
| | Potato | Moderate, absent |
| | | Abundant |
| | | Discolored |
| | | Starch destroyed |
| | | Grows at 37° C. |
| | | Grows in Cohn's sol. |
| | | Grows in Uschinsky's s |
| BIOCHEMICAL FEATURES | Liquefaction | Gelatin (4) |
| | | Blood-serum |
| | | Casein |
| | Milk | Acid curd |
| | | Rennet curd |
| | | Casein peptonized |
| | | Indol (5) |
| | | Hydrogen sulphide |
| | | Ammonia (6) |
| | | Nitrates reduced (6) |
| | | Fluorescent |
| | | Luminous |
| DISTRIBUTION | | Animal pathogen, epizo |
| | | Plant pathogen, epiphy |
| | | Soil |
| | | Milk |
| | | Fresh water |
| | | Salt water |
| | | Sewage |
| | | Iron bacterium |
| | | Sulphur bacterium |
| | | |

**Optimum Temperature.**—Not often used, but may furnish absolute evidence of the dissimilarity of two organisms. Not only the optimum but also the range of temperature, especially lower, should be determined.

**Pigment Production.**—The color and shade, the optimum medium and temperature for its production, limited to the area of growth, or diffused through the medium; its solubility in extracting agents, as alcohol, ether, chloroform, etc.; more than one pigment present, as shown by use of different extractives. In the case of the coccaceæ especially, the presence of pigment and its color and shade may be determined with ordinary agar; the growth is taken up with a platinum loop and spread on a piece of white glazed paper. Although the presence of pigment may be completely obscured on the agar, its presence is immediately evident on the paper.

**Ferment Action** (Cultural Evidence of).—The most important culturally are those causing fermentation of carbohydrates. Less important are proteolytic, diastatic, inverting, and rennin-like ferments.

*Fermentation of Carbohydrates.*—The sugars selected will depend on the organism to be identified as well as the medium selected as a basis for the addition of the sugar. The preparation and the use of indicators or titration to determine the production of acid and the use of fermentation tubes to demonstrate gas production have been described. The amount of gas is expressed in terms ot percentage, thus if the closed arm is half-full of gas, 50 per cent., etc. To determine roughly the proportion of carbon dioxide and of hydrogen, mark on tube quantity of gas produced, then fill the bulb with sodium hydroxide solution (10 per cent.) and insert a rubber stopper; the tube is inverted several times to mix and the gas again collected in the closed arm. The gas absorbed is carbon dioxide; the remainder is usually hydrogen. In the case of media containing coagulable proteins (as in milk, serum water, serum broth), acid production, if sufficient, is followed by coagulation; the coagulation is shown in solid media by precipitation or opacity. The ability to attack a carbohydrate may differ under aërobic and anaërobic conditions.

*Proteolytic action* is shown by gelatin liquefaction, liquefaction of Löffler's blood serum, digestion of milk. Prolonged incubation even for a month may be needed. The production of peptone may also be used for determining the digestion of albuminous media. Note odor and, if present, its character.

**Alkali Production.**—Determined by indicators or titration; note in milk cultures especially. An initial acidity may be noted, due to traces of a fermentable carbohydrate. The cultures may have to be incubated for one to three weeks. Milk on prolonged incubation may become translucent whether due to alkali production or to a proteolytic enzyme not determined.

**Sulphuretted Hydrogen.**—To demonstrate, use peptone water cultures to which is added 1 per cent. of a 1 per cent. solution of lead acetate or ferric tartrate; the precipitate which forms on mixing these turns black if sulphuretted hydrogen is produced.

**Nitrate Production.**—Grow in the following medium. Two solutions are necessary for the test.

| | |
|---|---|
| 1. Naphthylamin | 0.1 gm. |
| Distilled water | 20.0 c.c. |
| Acetic acid, 25 per cent. | 150.0 c.c. |

Dissolve the naphthylamin in the water by means of heat, cool, filter, and add the acetic acid.

| | |
|---|---|
| 2. Sulphanalic acid | 0.5 gm. |
| Acetic acid dil. 1 to 16 | 150.0 c.c. |

Keep them separate and mix in equal parts as needed.

To about 4 c.c. of the culture add 2 c.c. of the mixed solutions. The development of a pink color shows the presence of nitrates, the intensity of the color being proportional to amount present.

Similar reduction processes are seen in the decolorization of litmu some of the aniline dyes. The bacteria utilize the oxygen of the dyes, re them to their leuco-bases.

**Aromatic Products** (*Indol Production*).—The usual medium is peptone Two methods are available for testing: (1) Salkowski Method. To the add several drops of concentrated sulphuric acid or 1 c.c. of a 10 pe solution and then add 1 c.c. of a 1 to 10,000 sodium sulphite solution. color develops at the point of contact with the acid, which on shaking d throughout. (2) Ehrlich Method. This test is more constant and re To the culture add 1 c.c. of a 2 per cent. solution of paradimethylamin aldehyde in 95 per cent. alcohol and then add, drop by drop, concen hydrochloric acid until a red zone appears at the point of contact of the a and the peptone. Not more than 0.5 c.c. of the acid is required. On sta the zone deepens and widens. The color is soluble and the test should b firmed by shaking with chloroform, which dissolves out the color. The te indol are made after four to six days' incubation. Kligler[1] gives a good r of the subject.

**Cholera-red Reaction.**—Nitrite is produced by some bacteria from the present as an impurity in the peptone, and the red color appears on the ac of acid alone.

Both of the above tests are interfered with by the presence of a ferme carbohydrate.

**Vosges-Proskauer Reaction.**[2]—Cultures are grown for two or three days C. in either dextrose broth or in dextrose potassium phosphate broth in f tation tubes. Add 1 c.c. of a strong 50 per cent. solution of potassium h ide solution and leave at room temperature for twenty-four hours. A p reaction appears as an eosin pink color at the top of the tube, which is e to the air, due to the presence of acetyl-methyl-carbinol.[3] Boiling 1 to 4 the culture with 5 c.c. of a 10 per cent. caustic soda solution is a quick met obtaining the reaction. By the sugar alone, a yellow color is produced these conditions when the test is negative. Shaking or blowing into the t promote oxidation hastens the reaction.

Rettger[4] cultivates the organisms for twenty-four hours and then te using equal parts of 5 c.c. each, of culture and of 10 per cent. potassium hyd and incubates at 37° C. Frequent observations and shaking of the tubes the day are necessary lest the detection of weak carbinol producers be m He claims that decidedly positive reactions can always be obtained in eight hours.

**Toxin Production.**—The presence of a toxin is not usually elicited b tural methods; one exception is the demonstration of hemolysins by p with blood agar. The hemolysis is shown by a clearing about the colony.

For other methods of demonstrating the production of toxin, and the immunological reactions and animal tests see the appropriate sections o work.

*Chart.*—The accompanying chart is given as an example of a complete c study of an organism and the methods of using cultural reaction for c cation. The characteristics to be elicited will depend on the group of org studied. In the case of many of the organisms pathogenic for man and a cultural characteristics give us only a presumptive identification, the the identification depending on animal and immunological tests.

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[1] Jour. Infect. Dis., 1914, xiv, 81.

[2] Ztschr. f. Hyg., 1898, xxviii, 20.

[3] Prescott and Winslow, 1913, 3d ed., p. 181.

[4] Personal communication from Dr. Rettger.

# CHAPTER V.

# THE USE OF ANIMALS FOR DIAGNOSTIC AND TEST PURPOSES.

SUITABLE animals are necessarily employed for many microbiological purposes which may be summarized as follows: (1) To obtain a growth of varieties that for any reason grow with difficulty on artificial culture media, as in the case of tubercle bacilli, material suspected of containing the bacilli is injected into guinea-pigs, with the knowledge that, if present, although in too small numbers to be detected by microscopic or culture methods, the bacilli will develop in the animals' bodies, and thus reveal themselves. Certain microörganisms have not yet been grown on artificial media. This is true of few bacteria, of most protozoa, of many of the spirochetes, and of certain unknown infectious agents such as produce smallpox and Rocky Mountain spotted fever. (2) To cause an increase of one variety of organisms in a mixture and thus obtain a pure culture. An injection of sputum subcutaneously in rabbits may give rise to a pure pneumococcus septicemia or a pure tuberculosis. (3) To test virulence: Animals are used to test the virulence or toxin production of organisms, where, as in the case of diphtheria, we have very virulent, attenuated, and non-virulent bacilli of, so far as we know, identical cultural characteristics. Here the injection of a susceptible animal, such as the guinea-pig, is the only way that we can differentiate between those capable of producing diseases and those that are harmless. Still another use of the animals is to differentiate between two virulent organisms, which, though entirely different in their specific disease poisons, are yet so closely allied morphologically and in culture characteristics that they cannot always be separated except by studying their action in the animal body both with and without the influence of specific serums. In this way the typhoid and colon bacilli may be separated, or the pneumococcus and streptococcus. (4) To test the antitoxic or microbicidal strength of sera: Diphtheria antitoxin is added to diphtheria toxin and injected into guinea-pigs, and streptococcus immunizing serum is mixed with living streptococci and injected into the vein of a rabbit. (5) To produce antitoxic, bactericidal, or agglutinating sera for therapeutic and diagnostic use. (6) To obtain leukocytes for testing phagocytosis.

**The Inoculation of Animals.**—The inoculation of animals may be made by any one or more of the following methods:

1. Cutaneous. The material is rubbed into the abraded skin.
2. Intracutaneous. The material is injected into the skin. Important specific local reactions may be obtained by this method.

3. Subcutaneous. The substances are injected by means of a hypodermic needle under the skin, or are introduced by a platinum loop into a pocket made by an incision.

4. Intravenous. The substances are injected by means of a hypodermic needle into the vein. This is usually carried out in the ear vein of the rabbit. If rabbits are placed in a holder, so that the animal remains quiet and only the head projects, it is usually easy to pass a small needle directly into one of the ear veins, especially those running along the edge of the ear. If the ear is first moistened with a 3 per cent. carbolic acid solution, and then supported between the finger inside and the thumb outside, the vein is usually clearly seen and entered with ease, if a small, sharp needle is held almost parallel with the ear surface and gently pushed into it. When no holder is obtainable, the rabbit can be held by an assistant seizing the forelegs in one hand and the hind in another and holding the rabbit head downward, or the animal may be held between the knees of the operator, its body resting on the operator's apron.

5. Into the anterior chamber of the eye.

6. Into the body cavities. The peritoneal and less often the pleural cavities are used for microbal injection. The hypodermic needle is usually employed, less often a glass tube drawn out to a fine point. The needle or the pointed glass tube is gently pushed through the abdominal wall, moved about to be certain that the intestines have not been perforated and the fluid injected.

7. By inhalation. This method is carried out by forcing the animal to inhale an infected spray or dust.

8. By the trachea. This method is carried out by making an incision in the trachea and then inoculating the mucous membrane or injecting substances into the trachea and bronchi.

9. Through the intestinal tract by swallowing or by the passage of a rubber tube. Morphin may be given to prevent peristalsis.

10. Into the brain substance or ventricles after trephining, or when the parietal bones are thin, as in the guinea-pig and the rabbit, after making a tiny opening with the point of a small, heavy scalpel.

Mice, which are frequently inoculated at the root of the tail, are best placed in a mouse holder, but can be inoculated by grasping the tail in a pair of forceps, and then, while allowing the mouse to hang head downward in a jar, a glass plate is pushed across the top until only space for its tail is left.

Monkeys and apes are used for certain infections, such as syphilis and smallpox, where only they and man are markedly susceptible.

All these methods must be carried out with the greatest care as to cleanliness, the hair being clipped and the skin partially, at least, disinfected. The operator must be careful not to infect himself or his surroundings. After the inoculations the animals should be given the required food and kept in appropriate quarters. For food, rabbits and guinea-pigs usually require only carrots and hay.

When possible, all animals should be anesthetized during painful experiments.

**Autopsies.**—Autopsies should be made at the earliest moment possible, for soon after death some of the species of the bacteria in the intestines are able to penetrate through the intestinal walls and infect the body tissues. If delay is unavoidable, the animals should be put immediately in a place where the temperature is near the freezing-point. In making cultures from the dead bodies the greatest care should be taken to avoid contamination. The skin should be disinfected, and any dust prevented by wetting with a 5 per cent. solution of carbolic acid. All instruments are sterilized by boiling in 3 per cent. washing-soda solution for twenty minutes. Changes of knives, scissors, and forceps should be made as frequently as the old ones become infected. When organs are examined, the portion of the surface through which an incision is to be made must be sterilized, if there is danger that the surrounding cavity is infected, by searing with the flat blade of an iron spatula which has been heated to a dull red heat. Tissues if removed should be immediately placed under cover so as not to become contaminated. Sterile deep Petri plates are useful for this purpose.

When it is necessary to transport tissues from a distance, they should be wrapped in sterile cloths and sent to the point of destination as soon as possible. In warm weather they may be kept cool by surrounding the vessels which contain them with ice.

Animals rarely show the same gross lesions as man when both suffer from the same infection.[1] The cell changes, however, are similar, and, also, so far as we can test them, the curative or immunizing effects of protective serums.

Animals comparatively frequently undergo spontaneous infections with organisms similar to those producing disease in man. This is particularly so with the streptococcus-pneumococcus group, so one must be constantly on one's guard in working with such organisms.[2]

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[1] Smith, Theobald: Factors in Pneumonia of Lower Animals, Jour. Med. Res., 1913, xxix, 291.

[2] Holman, W. J.: Natural or Spontaneous Infection in Animals, Jour. Med. Res., 1916, xxxv, 152. (With bibliography.)

# CHAPTER VI.

# THE PROCURING AND HANDLING OF MATERIAL FOR MICROBIOLOGICAL EXAMINATION FROM THOSE SUFFERING FROM DISEASE.

A LONG experience has taught us that physicians very frequently take a great amount of trouble, and yet, on account of not carrying out certain simple but necessary precautions, make worthless cultures or send material almost useless for microbiological study.

In making cultures from diseased tissues various procedures may be carried out, according to the facilities which the physician has and the kind of information that he desires to obtain. From the dead body culture material should be removed at the first moment possible after death. Every hour's delay makes the results less reliable. From both dead and living tissues, the less the alteration that occurs in any substance between its removal from the body and its examination and inoculation upon or in culture media or animals, the more exact will be the information obtained. If the material is allowed to dry, many microbes will be destroyed in the process, and certain forms which were present will be obliterated or, at least, entirely altered in the proportion which they bear to others. If possible, therefore, smears should be made and culture media should be inoculated directly from the patient or dead body. For the latter purpose a microbiologist should take the most suitable of the culture media to the bedside or autopsy table. Such a list of media, if fairly complete, would comprise nutrient bouillon alone and mixed with one-third its quantity of ascitic fluid, slanted nutrient agar, slanted agar streaked with rabbit or human blood, firmly solidified slanted blood serum and slanted ascitic glucose agar. Additional media will be necessary for special purposes, such as the isolation of typhoid or tetanus bacilli. If only one variety of media is to be used, the solidified blood serum is most useful for parasitic bacteria, and this can be easily carried by the physician and inoculated by him, even if he is not very familiar with bacteriological technic. In the first place some of the infected material should always be smeared on a couple of clean slides or cover-glasses and fixed moist in methyl alcohol or allowed to dry in the air. These can be stained and examined later, and may give much valuable information. For special media in isolation see chapter on Media.

The material must be obtained in different ways, according to the nature of the infection.

For the detection of the bacteria causing septicemia we are met with the difficulty that there are apt to be very few organisms present in the blood until shortly before death. It will therefore be almost useless to take only a drop of blood for cultures, as even when present there may not be more than eight to ten organisms in a cubic centimeter. If cultures are to be made at all, it is therefore best to make them correctly by taking from 5 to 20 c.c. of blood by means of a sterile hypodermic needle or a suitable glass tube armed with a hypodermic needle, from the vein of the arm after disinfecting the skin with tincture of iodin. To each of five different tubes containing bouillon we add 1 c.c. of blood, and to a flask containing 100 c.c. we add 5 c.c. We have made by this mixture of blood and bouillon a most suitable medium for the growth of all bacteria which produce septicemia, and at the same time have added a sufficient quantity of blood to insure us the best possible chance of having added some of the bacteria producing the disease. We also add to each of several tubes of melted nutrient agar, at 40° C., 1 c.c. of blood and pour the mixture into Petri plates, so as to indicate roughly the number of organisms present by the number of colonies developing. When blood must be carried to a distance, clotting should be prevented by having in the test-tube sufficient 10 per cent. solution of sodium citrate, bile, or ammonium oxalate to prevent clotting.

From wounds, abscesses, cellulitis, etc., the substance for microbiological examination can, as a rule, best be obtained by means of a syringe, or when the lesion is opened, by small rods armed with a little absorbent cotton. A number of these swabs can be sterilized in a test-tube and so carried. The swab is inserted in the wound, then streaked gently over the oblique surface of the nutrient agar in one tube, over the blood serum in another, and then inserted in the bouillon. Finally, either at the bedside or in the laboratory, material is thinly streaked over the surface of nutrient agar contained in several Petri dishes. We inoculate several varieties of media, with the hope that one at least will prove a suitable soil for the growth of the organisms present. From surface infections of mucous membranes, as in the nose, throat, vagina, etc., the swab, again, is probably the most useful instrument for obtaining the material for examination. The greatest care, of course, must be used in all cases to remove the material for study without contaminating it in any way by other material which does not belong to it. Thus, for instance, if we wish to obtain material from an abscess of the liver, where the organ lies in a peritoneal cavity infected with microbes, one must first absolutely sterilize the surface of the liver by pressing on it the blade of a hot iron spatula before cutting into the abscess, so that we may not attribute the infection which caused the abscess to the germs which we obtained from the infected surface of the liver. From such an organ as the uterus it is only with the greatest care that we can avoid outside contamination, and only an expert microbiologist familiar with such material will be able to eliminate the vaginal from the uterine microbes.

A statement of the conditions under which materials are obtained should always accompany them when sent to the laboratory for examination, even if the examination is to be made by the one who made cultures. These facts should be noted, or otherwise at some future date they may be forgetten and misleading information sent out. The work of obtaining material for examination without contamination is at times one of extreme difficulty. It simply must be remembered that if contamination does take place our results may become entirely vitiated, and if the difficulties are so great that we cannot avoid it, it may simply mean that under such conditions no suitable examination can be made. Where the substance to be studied cannot be immediately subjected to cultures or animal inoculations, it should be transferred in a sterile bottle as soon as possible to a location where the cultures can be made. If for any reason delay must take place, the material should at least be put in a refrigerator where cold will both prevent any further growth of some varieties of microbes and lessen the danger of the death of others.

In obtaining samples of fluid, such as urine, feces, etc., the bottles in which they are placed should always be sterile, and, of course, no antiseptic should be added. It is necessary clearly to explain this to the nurse, for she has probably been instructed to add disinfectants to all discharges. Disinfected material is, of course, entirely useless for complete microbiological investigations. It cannot be too much emphasized that materials which are not immediately used should be sent to the laboratory as quickly as possible, for in such substances as feces, where enormous numbers of various kinds of microbes are present, those which we seek most, such as the typhoid bacilli, frequently succumb to the deleterious products of the other microbes present. Even when abundantly present, living typhoid bacilli may entirely disappear from the feces in the course of twelve hours, while at other times they may remain for weeks. These differences depend on the associated organisms present, the chemical constitution of the feces or urine, and the conditions under which the material is obtained. Water and milk rapidly change in their bacterial content if not kept under 40° F.

For obtaining fluid for agglutination and other purposes, blister fluid is valuable. A blister can be raised quickly by placing a piece of blotting paper moistened with a little strong ammonia on the skin and covering with a watch-glass, or one may be more slowly formed by a cantharides plaster.

In making a microbiologic examination of sputum we carry out the procedure, when feasible, of what is known as *sputum washing*. Some of the associated bacteria found in the expectoration come from the diseased areas of the lungs, while others are merely added to the sputum as it passes through the mouth or are developed after gathering. To endeavor to separate the one from the other we wash the sputum. The first essential is that the material is to be washed within a few minutes, and certainly within an hour after being expectorated. If a longer time is allowed to intervene, the bacteria from the mouth will penetrate into the interior of the mucus, and thus appear as if they came from the lungs. Sputum treated twenty-four hours after its expectoration is useless for examining for anything except the tubercle bacillus. A rough method is to pour some of the specimen of sputum to be examined into a convenient receptacle containing sterile water, and withdraw, by means of a sterilized platinum wire, one of the cheesy masses or thick "balls" of mucus. Pass this loop five times through sterile water in a dish; repeat the operation in fresh water in a second and third dish. Spread what remains of the mass on cover-glasses and make smear preparation; stain and examine. With another mass inoculate ascitic bouillon in tubes and agar in plates.

When we wish to exclude thoroughly mouth bacteria, a lump of the sputum raised by a natural cough is seized by the forceps and transferred to a bottle of sterile water and thoroughly shaken; it is then removed to a second bottle of bouillon and again thoroughly shaken. From this it is passed in the same way through four other bottles of bouillon A portion of the mass is now smeared over cover-glasses, and the rest inoculated in suitable media, such as agar in Petri dishes, and ascitic fluid bouillon in tubes. If desired, the bacteria washed off in the different washings are allowed to develop.

**Routine Technic Carried Out at Laboratory when Thorough Examination Required.**—As has just been indicated, the microbiological examination proceeds somewhat differently, according to the information needed. As full a history of the case as possible should be sent with the material. Especially the stage of the disease at which the material to be examined was gathered should be stated. This aids the examiner in deciding what method to use in his examination, and how to judge of his results. Beginning infections usually give a different microbiologic picture from later stages. Then secondary infections must be considered. When, as is the case with most clinical material, definite knowledge in regard to the presence or absence of a particular organism is desired, the culture media are used which are known to be most suitable for the organisms sought, such as Löffler's blood medium for the diphtheria bacillus, Petroff medium for the tubercle bacillus, and so on. These media have been already given in the chapter on Culture Media. When a complete examination is needed in order to determine unknown organisms, as is generally the case with autopsy material and sometimes with clinical, the procedure may be as follows:

1. At the autopsy table the routine cultures and smears are made as described above.
2. Material from the different parts is secured under aseptic precautions in sterile receptacles and taken to the bacteriological laboratory. The receptacles should be surrounded by ice if the laboratory is at a distance.
3. A smear from each part is stained and examined in order to determine in some measure the kind and number of microbes present, so we may more wisely select suitable culture media if other than those already used are needed, and may make the right culture dilutions if these are necessary. Exceptionally, cultures are made before smears are taken.

**Use of Gram's Stain.**—Gram's stain gives more information as to the kind of germs present than any other one stain, so when possible this stain should be used. Other stains, however, may help, if for any reason Gram's is not at hand; and smears made from blood or from suspected syphilitic material should be stained by Giemsa's method or an equivalent (see Staining Methods for formulas of stains). Of course, if one is looking for a special organism, the special stain for that organism should be used.

A Gram-stained smear may show all Gram-negative or all Gram-positive microbes or a mixture of the two, or it may show a number only partially stained (Gram-amphophile).

The following points must be remembered in using the stain and in interpreting results:

(*a*) The smears should be thin and evenly spread.

(*b*) The staining solutions should be fresh (anilin-water-gentian violet lasts about three weeks).

(*c*) Controls, fresh cultures (about twenty-four hours old) of a Gram-negative and a Gram-positive bacterium, should be used usually on the same slide with the smear to be examined.

(*d*) If there is much albumin in the suspected material less heat should be used in fixing.

(*e*) If the urine is very acid, the results may not be good.

(*f*) Mix urinary sediment with egg albumen, better to fix it; and wash out urinary salts with tap-water and stain.

(*g*) Too much dependence should not be placed upon the finding of Gram-negative microbes in tissues, since organisms which in pure young cultures may be positive to Gram, show forms as they grow older both in tissues and in cultures, intermediate between the positive and negative, as well as a varying number of negative forms.

If the smears show only Gram-negative organisms, the material probably contains one or more of the following:

| | | | |
|---|---|---|---|
| Gram-negative bacilli. | B. coli group. | Most frequently from intestinal tract. | Most frequently found, and some indication of their presence in history. |
| | B. typhosus group | | |
| | B. dysenteriæ group. | | |
| | B. proteus. | | |
| | B. mucosus (capsulatus). | | |
| | B. pyocyaneus. | Most frequently from chest contents. | |
| | B. influenzæ group. | | |
| | B. pertussis group. | | |
| | B. fusiformis. | | |
| | B. mallei. | Less frequently found, and generally a marked indication of their presence in history. | |
| | B. edematis (malignant edema). | | |
| | B. of symptomatic anthrax. | | |
| | B. pestis. | | |
| | B. of Morax-Axenfeld. | | |
| Gram-negative cocci. | Micrococcus meningitidis. | Generally marked indication of their presence in history. | |
| | Micrococcus catarrhalis. | | |
| | Micrococcus gonorrheæ. | | |
| | Micrococcus melitensis. | | |
| Gram-negative spirilla. | S. choleræ and allied forms. | Marked indication of presence of first form in history. | |
| | Mouth spirals. | Unimportant, unless indicated in history, when Tr. pallidum or Sp. recurrentis, respectively, should be looked for. | |
| | Tr. pallidum. | | |
| | Sp. recurrentis. | | |

Old forms of any of the Gram-positive or Gram-amphophile organisms.

If only Gram-positive organisms are demonstrated, the material may contain one or more of the following:

| | | |
|---|---|---|
| Gram-positive bacilli. | B. diphtheriæ group.<br>B. tetani (not often demonstrated in smears from lesion).<br>B. tuberculosis group.<br>B. lepræ group.<br>B. anthracis group.<br>B. welchii and some other intestinal anaërobes. | Generally marked indication of their presence in history. |
| Gram-positive cocci. | Staphylococcus group.<br>Streptococcus group (including pneumococcus and its variety, pneumococcus mucosus).<br>Micrococcus tetragenus. | Some indication of their presence in history. |
| Gram-positive spirilla. | None. | |
| Higher bacteria. | Nocardia.<br>Actinomyces. | |

Yeasts and moulds, certain forms.

If organisms partially stained by Gram's method are demonstrated, the material may contain one or more of the following:

| | |
|---|---|
| Amphophile. | Moulds.<br>Yeasts.<br>Protozoa.<br>Slightly known cocci and bacilli. |

Certain slightly known streptococci and bacilli, older forms of any of the Gram-positive organisms.

**Regional Distribution of Microörganisms.**—From the different parts of the body the following more important organisms are found in the order of their probable frequency.

| | | | |
|---|---|---|---|
| Fluids from serous membranes. | Meningeal (cerebrospinal). | Micrococcus meningitidis.<br>Streptococcus (including pneumococcus) group.<br>B. influenzæ group. | Fluid generally cloudy with many leukocytes. Poliomyelitis fluid nearly clear, by transmitted light ground glass appearance. |
| | | B. tuberculosis group. | Fluid generally clear. |
| | Pericardial and pleural. | Streptococcus (including pneumococcus) group.<br>B. mucosus (capsulatus) group. | Fluid may be cloudy. |
| | | B. influenzæ (hemoglobinophilic bacilli) group.<br>B. tuberculosis group. | Fluid generally clear. |
| | Peritoneal. | B. coli group.<br>Streptococcus group.<br>B. tuberculosis. group.<br>Yeasts, moulds, amebæ. | |

| | |
|---|---|
| Lungs. | Streptococcus (including pneumococcus) group.<br>Micrococcus (including staphylococcus and diplococcus) group.<br>B. mucosus (capsulatus).<br>B. influenzæ group.<br>B. tuberculosis group.<br>B. pertussis group.<br>Nocardia. Yeasts, moulds, amebæ. |
| Nose and throat. | Streptococcus group.<br>B. diphtheriæ group.<br>B. influenzæ group.<br>B. mucosus group (micrococcous group).<br>B. fusiformis.<br>B. tuberculosis.<br>Yeasts, moulds, amebæ.<br>Tr. pallidum and other spirochetes. |
| Sputum. | Same organisms as those found in the lungs, nose and throat. |
| Eye. | Streptococcus (including pneumococcus group).<br>Group of hemoglobinophilic bacilli.<br>Gonococcus group.<br>B. Diphtheriæ group.<br>B. Morax-Axenfeld group.<br>B. tuberculosis group.<br>Yeasts and molds. |
| Feces. | B. coli (including B. alkaligenes and B. lactici).<br>B. typhosus group.<br>B. dysenteriæ group.<br>Gram-positive anaërobes.<br>Yeasts, molds, amebæ.<br>B. tuberculosis.<br>Vibrio cholera group.<br>B. pestis.<br>B. anthracis.<br>Many forms whose importance has not been worked out. |
| Urine. | B. coli group.<br>Streptococcus (kidney).<br>M. gonorrheæ.<br>B. typhosus.<br>B. tuberculosis.<br>Moulds, flagellates. |
| Pelvic organs. | M. gonorrheæ.<br>Streptococcus.<br>B. tuberculosis.<br>Treponema pallidum.<br>Certain anaërobes.<br>Many other forms probably much less important. |
| Blood. | Streptococcus (including pneumococcus) group.<br>B. typhosus group.<br>Trypanosome group.<br>Group of malarial organisms.<br>B. anthracis.<br>Sp. recurrentis. |
| Brain. | Micrococcus group.<br>Strepto-pneumococcus group.<br>Group of hemoglobinophilic bacilli.<br>B. tuberculosis group.<br>Tr. pallidum.<br>Malarial organisms.<br>Rabies organisms.<br>Yeasts, moulds, etc. |

**Choice of Media.**—The following media should be used for the accompanying reasons:

Nutrient broth, for motility, morphology, and arrangement (chains, groups, etc.).

Potato for color and abundance of growth.

Peptone broth for indol.

Fermentation tube for anaërobes, acidity and gas.

Nutrient agar and gelatin.
- (*a*) Poured plates for isolated colonies (dilutions according to the number of organisms seen in smears). (Blood-agar if pneumococcus or streptococcus indicated.)
- (*b*) Streaked plates for surface colonies. (Blood-agar if influenza bacilli are indicated.)

## CHAPTER VII.

# THE RELATIONSHIP OF MICROORGANISMS TO DISEASE AND THE RESISTANCE OF THE HOST TO MICROBAL INFECTION.[1]

In preceding chapters we have considered the growth of microorgans, for the most part, in dead organic substances. Now we must consider their growth on or in the living host and the effect of their growth and their products upon the health of the host. Although the living body serves as a food mass for the growth of certain microörganisms, it is not a passive source of food supply. The protoplasm of the cells of the body, as well as substances in the blood and lymph and other secretions, are relatively antagonistic to the growth of microörganisms. Under the influence of health or response to infection these germicidal substances may increase through cell activities, while the efforts of strain, hunger and acute poisoning may cause them to diminish.

**Parasitic and Pathogenic Microörganisms.**—Certain microörganisms have become adapted to growth upon the physiological exterior of the body: that is, upon the mucous membranes of the upper respiratory tract and of the alimentary canal, upon the skin and in the opening of skin glands. Whatever slight antagonistic action the local secretion may possess is compensated for by adaptation of the microörganisms. The adaptability of microörganisms to the sites mentioned is primarily dependent upon the temperature, food and reaction (acidity or alkalinity) requirements of the microörganisms. As far as we know this multiplication is usually without effect upon the health of the host. Under certain conditions, irritation of the mucous membranes may result, and to some extent small numbers of the microörganisms may find their way into the deeper tissues or in the blood and lymph only, to be destroyed by the defensive mechanism of the body. Such microörganisms, as long as they do not produce inflammation of the mucous membranes, or invade, multiply and injure the deeper tissues or organs, exist for these individuals as harmless parasites or commensals (p. 51). The ability to harm the host is inherent in many of the varieties of microörganisms normally present in the areas noted, that is, they are potentially pathogenic or able to produce disease if the host's resistance is lowered or if transferred to others who are more susceptible. Certain types of microörganisms which are parasitic but not generally present, have pathogenic characteristics much more highly developed. These types are, as a rule, the etiological agents in the more specific or more highly differentiated types of disease which occur more rarely or which find no nearly related types present as less harmful parasites in the invaded host. Because their pathogenicity is more highly developed or they find no immunity, they cause disease with greater regularity when they reach a host. A varying proportion, however, because of the resistance of the host fail to develop disease, but the microörganism may multiply on the physiological exterior of the body existing under these conditions as harmless parasites (See below under Carriers.) Pathogenicity, therefore, is relative.

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[1] For more detailed discussion and bibliography of the subject matter of this and the following chapters, reference is made to the following:

I. G. Adami: Principles of Pathology, vol. i. H. Zinsser: Infection and Resistance. J. A. Kolmer: Infection, Immunity and Specific Therapy. P. Ehrlich: Studies in Immunity (translated by C. Boldnan). J. Bordet: Studies in Immunity (translated by F. P. Gay). E. Metchnikoff: Immunity in Infective Diseases (translated by F. G. Binnie). R. Kraus and C. Levaditi: Handbuch der Technik und Methodik der Immunitätsforschung. H. Zinsser, J. G. Hopkins and R. Ottenberg: Laboratory Course in Serum Study. H. G. Wells: Chemical Pathology. S. Arrhenius, Immunochemistry. H. Bechold: Colloids in Biology and Medicine (translated by J. G. M. Bullowa). Also special sections in Kolle and Wassermann: Handbuch der Pathogenen Microörganismen.

**Disease-producing Power or Pathogenicity.**—The disease-producing power or virulence of microörganisms is susceptible to variation. Virulence is the term we employ to indicate the relative infectious ability of a microörganism or its capacity to overcome the protective mechanism of the host. This may be due to the capacity of the microörganism to produce poisons or toxins ("toxicity") or to its aggressive or invasive ability or to both. Both these factors are susceptible to variation. Under natural conditions the virulence of a microörganism tends to be increased until it reaches its limit by the rapid successive transfer from host to host. The invasive virulence is highest as it leaves the body and tends to decrease the longer the period since its exit, and its exposure to the modifying effect of a different environment.

In the laboratory the invasive capacity and to a much less extent the toxicity of microörganisms can be easily modified. Invasive virulence may be decreased by cultivation upon artificial culture media, by exposure to high temperature, which, however, is not sufficient to kill, by dessication, by exposure to light, by the action of small amounts of antiseptic substances, and so on. Invasive virulence may be increased by the addition of body fluids to culture media, by passage through successive animals of the same species or by cultivation in collodion sacs placed in the tissues or cavities of living animals. An increased virulence for one species is not necessarily associated with an increase for other species. In some cases the increase for one is associated with a lowering for another.

The toxin-producing capacity of microörganisms remains relatively constant even after cultivation on artificial culture media for long periods of time. The strain of diphtheria bacillus that we use to produce toxin has been grown in nutrient broth for twenty-five years.

Different strains of one and the same type of bacterium vary widely in their ability to produce toxin, but the exhibition of the maximum ability of the individual strains rests primarily upon the suitability of cultural conditions.

Because the invasive capacity is the important factor with most pathogenic bacteria the quantitative term "virulence" is limited by some to denote the degree of invasive power only and toxicity employed for toxin production.

**Influence of Quantity on Infection.**—If microörganisms are extremely virulent and no antibodies are present in the host, infection may result when very few or even one gains entrance to the body. When less virulent the introduction of increased numbers may compensate for the lower virulence. In the latter case infection may result if the body resources immediately available are exhausted in destroying a smaller or greater part of the microörganisms introduced, thus allowing those that survive to multiply and as the generations develop to gradually increase in virulence.

**Mixed and Secondary Infections.**—An infection may from the start be due to more than one variety of microörganism. This is most commonly observed in infections of wounds and where the skin and mucous membranes are involved, or the extension of infection from these membranes.

Secondary infections, that is, the implantation of one or more varieties of microörganisms upon an existing infection, are common. To a large extent this is due to the lowering of tissue resistance by the initial organism. An example of such secondary infection is seen in the multiplication of streptococci in the throat or the occurrence of otitis, adenitis or even general blood infection due to this bacterium in a patient whose resistance has been lowered by scarlet fever. Secondary infection is especially common in infections of the skin and of the respiratory or intestinal tracts because of the normal presence of many varieties of bacteria or of the addition of new forms from outside contamination. These bacteria under normal conditions exist as parasites even though they may possess some degree of virulence. With the reduction of resistance or actual destruction of the skin or membranes due to inflammation one or more varieties may be able to cause secondary infection. Thus in smallpox, staphylococci secondarily infect the skin lesions; in influenza, influenza bacilli, streptococci or pneumococci commonly cause the secondary pneumonias and in intestinal diseases the colon types may aid in the extension of an inflammation. Complicating lesions due to extension of infection from the respiratory or intestinal tract are commonly mixed infections due to the invasion by the primary and a secondary infecting organism or at times to the invasion of two or more secondary organisms alone. In fact, in many diseases the secondary infection when once established may be much the more important, not only in prolonging and extending the infection but also as a cause of death. Where two or more infectious agents are present and a general blood invasion follows, as a rule, only one type will have invaded the blood stream. In the case of secondary infections the invading type is frequently the secondary organism.

Another factor in mixed or secondary infections is the influence of the microörganisms one upon the other. One may aid the other as, for example, the presence of aërobic bacteria in a wound promotes the development of the tetanus bacillus and its spores, probably by absorbing uncombined oxygen, in the presence of which B. tetani cannot multiply. One may injure the other as, for example, the pyogenic cocci in anthrax.

**Protective Powers of the Host.**—In the production of disease the degree of protective power of the host is no less important than the degree of pathogenicity of microörganisms. Upon the degree of development of this protective mechanism depends the resistance or immunity of the host.

Immunity or resistance to infection depends upon four general defences: (1) The mechanical barrier offered by the coverings of the physiological exterior of the body, viz., the skin and mucous membranes; (2) the mechanical and chemical action of secretions secreted by the various glands connected with the physiological exterior of the body; (3) the chemicophysical action of substances in the blood or lymph which bathe the body cells (ferments or antibodies); (4) the ability of certain cells to engulf and destroy microörganisms (phagocytosis).

*Mechanical Barriers to Infection.*—The skin and to a somewhat less extent the mucous membranes form very efficient barriers to infection. The thickness of the membrane and the relatively small number of glands, and the absence of crypts or pockets favor protection. Microörganisms may pass through these membranes following very slight injury. This may be so slight that the membranes are apparently intact.

In both instances no signs of inflammation may develop at the site of entry. Should the slight injury in the former instance heal, in neither case would any indication be left, as to the point of passage. The opening of the glands as well as crypts or folds offer places for the accumulation of bacteria. These may multiply and cause injury to the lining epithelium thus making a breach for invasion. The cilia of the healthy cells sweeping the inspired microörganisms outward toward the mouth aids in protecting bronchioles and alveoli.

**Chemical and Mechanical Action of Secretions of Mucous Membranes.**—These secretions when discharged on the surface aid mechanically by washing away the microörganisms and the toxic products of their action on the tissues. The secretions also restrain the growth or actually kill many varieties of microörganisms because of their constituents or to a less extent because of the presence of antibodies in the serous element of the secretions.

The saliva aids in preventing infections as is evidenced by the development of a fetid condition of the mouth when this secretion is diminished. The gastric juice is strongly bactericidal because of its content in free hydrochloric acid. In spite of this, however, many microörganisms pass through the stomach because of the protection by food particles as well as their greater resistance to the action of acid. The intestinal secretions are probably without effect on most microörganisms. The bile, however, is moderately bactericidal, this action being materially greater when the secretion is alkaline in reaction.

**Ferments or Antibodies.**—The ferments present in body fluids, which are the basis of the general body metabolism may act upon microorganisms destroying them and digesting them and their products. Certain substances of a ferment character known as antibodies may be present in the blood of normal individuals. These antibodies may destroy microörganisms or neutralize their poisons or toxins. Should infection occur, and be not too severe, their production is stimulated and their increase is an essential factor in the limitation and in the recovery from infection or disease, as well as (see below) in the protection of the individual from a recurrence of the same infection or disease.

**Cellular Activity or Phagocytosis.**—This ability to ingest and destroy microörganisms is possessed by two types of cells; the leukocytes, especially the polynuclear type and the endothelial cells. The activity of these cells is an essential protection of the body from infection.

Although phagocytosis is a cellular activity it is not independent of the antibodies, the latter are necessary in most instances to prepare the bacteria for ingestion. The mechanism of this phenomenon in relation to the prevention and to the limitation of infection is given in detail in a later chapter.

**Immunity, Varieties and Factors Involved.**—Immunity to one or more types of infection or disease is normally possessed by many individuals. This is termed natural immunity. All the factors discussed above underlie this type of resistance. Such natural immunity may be general or specific in character. The latter is evidenced by the resistance possessed by some individuals to certain specific diseases. Such special resistance depends not only upon the general factors given but also upon the possession of antibodies which are active against the specific causative organism or its toxins. This is especially striking in the natural immunity to diphtheria which is due to the presence of antibodies that can neutralize the toxins of the bacillus.

Immunity may be "acquired" either as the result of natural infection or disease or by the artificial production of a mild form of the disease or by the injection of the specific microörganism or its products. In this type of immunity the stimulation of the production of antibodies is probably the essential factor in the subsequent immunity. This stimulation may be followed not only by an increased content of antibody but also by an ability of the cells to more quickly and more freely respond to similar stimuli.

Acquired immunity may be "active," that is, due to the individual's own response. It may be "passive" through the injection of blood or serum containing antibodies from, and produced by another individual.

Although the terms natural and acquired may be employed for general purposes of discussion as defined, the definitions given are not altogether satisfactory. Thus, there is an increased general and specific resistance to disease as individuals grow older without regard to recovery from any disease or other demonstrable reason for this increase. This is most striking as regards diphtheria. Although this immunity is natural in one sense in another it is distinctly acquired. Against certain infections, this is due probably to the stimulation resulting from the passage through the mucous membranes of small numbers of microörganisms. The apparent lack of virulence of many respiratory and intestinal bacteria in persons in thickly populated regions is frequently due to an immunity developed in this way. Against others the cause of the development of immunity is wholly unknown.

Natural immunity may be further qualified as "inherited," "racial," "species," "familial," "individual." Examples are given in the following paragraph:

**Susceptibility to Infection.**—This is the reverse of immunity and is due to the absence or suppression of the activity of the factors which underlie immunity. Susceptibility may be general. This involves the absence or suppression of one or more or all these factors; or it may be specific, in which case there is a susceptibility to a specific disease due most probably to the absence of protective antibodies.

Susceptibility may be "natural" or "acquired" or may be "inherited," "racial," "species" or "individual."

Man as a species has an inherited susceptibility to the distinctively human diseases such as gonorrhea, typhoid fever, etc., whereas other species have an inherited immunity to these infections. Each species has a more or less distinctive susceptibility or immunity, thus the horse is susceptible to glanders but immune to tick fever, cattle are susceptible to tick fever but immune to glanders. Such types of susceptibility depend upon the adaptation of microörganisms to species peculiarities of cellular structure and metabolism. The immunity is the absence of such adaptation of the microörganisms.

Special peculiarities in relation to mutual affinity of cells and bacterial products are also a factor. Thus in the absence of such affinity, the cells cannot be harmed. An affinity may exist, but if the cell is not susceptible to injury the bacterial product is digested as though it were any inert substance. Such cellular peculiarities probably underlie the susceptibility of man to tetanus toxin and the immunity of the hen.

Racial immunity and susceptibility are shown by the susceptibility of ordinary sheep to anthrax, whereas Algerian sheep are immune. Racial immunity or susceptibility in man is probably largely dependent upon exposure of the race to infection. An example is the relative resistance of civilized races to tuberculosis whereas aboriginal races not only are more susceptible to infection but also show a higher mortality rate.

Young infants show a relative immunity to certain diseases such as diphtheria, measles, typhoid fever, etc. To a large extent this is due to passive transfer of antibodies from an immune mother. Susceptibility develops later because these are exhausted and not replaced by the cells of the child. Certain diseases, however, are essentially diseases of childhood as for instance ringworm of the scalp.

Susceptibility may be acquired for some diseases by depression of the general physiological activities of the body. This may result from overwork, overstrain or worry, inadequate rest or sleep, improper environment, lack of fresh air and sunlight, exposure to cold and wet, malnutrition due to insufficient or improper food, excesses in eating or use of alcohol, disease, organic or infectious, and trauma. Tuberculosis and typhoid fever are examples.

Certain types of individual susceptibility are of special interest because one attack of a disease seems to confer not an immunity but an actual predisposition toward later attacks. Two examples are pneumonia and erysipelas. Recurrent attacks may be due to the failure to develop immunity, but another and more probable factor must also be considered, viz.: recurrence of the disease due to a different variety of the same microörganism. Thus, the first attack of pneumonia may have been due to one type of pneumococcus only to which, the person develops an immunity and the latter attack be due to another type. The recurrent attacks would depend therefore primarily on an individual tissue or organ susceptibility which, in addition, might be increased through the damage done by the previous disease.

**Other Factors in Infection.**—We have considered in the previous paragraphs the relation of the virulence and number of microörganisms to the general factors of resistance offered by the host. Other factors, the portal of entry of microörganisms, the site of election for multiplication and the bearing upon this special tissue immunity or susceptibility are equally important in determining whether infection will occur.

**Tissue or Organ Susceptibility or Immunity.**—The general facts concerning the protective coverings, the skin and mucous membranes, as well as the protection afforded by cilia and also by the various secretions of the mucous membranes have already been considered. The blood and lymph owe their protective power to antibodies and phagocytic cells and serve as the barrier to extension of bacteria from without to tissues and organs open only to infection by these routes (hematogenous or lymphatic). The tissues and organs themselves are protected by the tissue fluids (lymph) and such added phagocytic cells as they possess. Although the general protection of the tissues and organs is basically similar very different susceptibility may be demonstrable even of the same type of bacterium.

Thus the diphtheria bacillus may infect the mucosa of the pharynx and extend frequently to the larynx and bronchus, but only very exceptionally does it cause inflammation of the esophagus. Drinking of milk containing virulent streptococci will cause a septic sore-throat but no evident infection of the gastro-intestinal tract. Similar peculiarities are noted in relation to portal of entry and site of election (see below). The muscle tissue enjoys an unusual freedom from infection.

**Portal of Entry or Route of Infection.**—Many microörganisms have definite portals of entry, to which they are adapted. If introduced by another mode they fail usually to cause infection of disease or at most only a transient local inflammation. Thus virulent cholera vibrios when swallowed cause the disease cholera, but injected into the tissues cause only a local inflammation. The typhoid bacillus likewise enters normally by the mouth, passes through the mucous membranes to the blood and causes a general infection, yet the introduction into the subcutaneous tissues would probably cause only a local lesion. The tetanus bacillus when introduced into the subcutaneous tissues may cause tetanus, yet taken by mouth is harmless, in fact the bacillus frequently leads a parasitic existence in the intestinal canal. The more definitely an organism is adapted to the production of a specific clinical disease the more sharply it is limited to one mode of entry. With some bacteria the type of disease produced will depend on the mode of entry, thus the plague bacillus entering through the skin causes bubonic plague, whereas, entry by the respiratory tract gives rise to pneumonic plague. It is probable that another factor is the adaptation of a germ to certain tissues. The germs from cases in an epidemic of the bubonic type would be less likely to cause pneumonia than the reception of the same number of germs from a case in an epidemic of the pneumonic variety.

**Site of Election: Selective Localization and Toxin Affinity.**—Having passed their normal portal of entry, should they be sufficiently virulent to overcome the factors of resistance given above, microörganisms select certain areas for localization and multiplication following which infection or disease results. Localization depends on tissue and organ immunity and susceptibility (see above) and differs widely with various types of microörganisms. It depends to some extent upon the mode of entry as in plague (see above). In some diseases the site selected is directly at or connected with the portal of entry. Thus the diphtheria bacillus in the pharynx or larynx, the dysentery bacillus in the intestine, etc. The selective localization is most striking in diseases where the microörganisms invade the blood stream to localize later. Thus the meningococcus selects the meninges; the virus of poliomyelitis, the motor areas of the central nervous system; the typhoid bacillus, lymphoid tissue, etc. Evidence has been advanced as already noted that races of one and the same microörganism may differ in their selective action, thus certain strains of the spirochete of syphilis injected into rabbits appear to select the ocular tissues for localization. Certain streptococci show definite tendencies to localize at the roots of teeth or in the joints or in the heart valves. Rosenow claims that the elective affinity of an individual strain breeds nearly true, when injections of rabbits are carried out with these strains. There is undoubtedly a tendency to do this. To some extent, the "screening" out of bacteria by or small emboli in the capillary circulation influences localization in individual cases, especially in relation to relative organ susceptibility. Thus the tubercle bacillus may localize in any organ if its resistance be low, but localize by preference in the lung. In the latter, it again selects by preference the apices. The latter would seem to be influenced by circulatory conditions, as depending on posture of the host, the bacillus selects the most elevated apex, that of the upper lobe in man, that of the dorsal lobe in quadrupeds.

The specific toxins of microörganisms show similar selective affinities, thus the tetanus toxin when absorbed affects chiefly the central nervous system, while the diphtheria toxin, acts widely on epithelium, connective tissue, and certain nerve centers.

**Types of Infection.**—"Exogenous" infection refers to the entry of microörganisms from without. "Endogenous" infection, to infection by microörganisms existing as simple parasites but which because of a temporarily lowered resistance of the host are able to initiate an infection. These two types of infection are frequently not differentiable. Infection may be local or general. The former may be at or remote from the portal of entry, in the latter case due to hematogenous or lymphatic transfer. When an invasion of the blood stream occurs but there is no evidence of active multiplication the condition is spoken of as a "bacteremia." This is of fairly common occurrence especially in the case of more severe local infections. When the pus-producing bacteria invade the blood and localize at different points causing foci the condition is called a "pyemia." When the blood invasion is followed by multiplication in the blood stream a "septicemia" is present. It is sometimes difficult to decide whether a case is a bacteremia or a septicemia.

When bacteria remain localized but the body cells are being injured by the absorption of the toxins produced a "toxemia" exists. The term sapremia is used to indicate the effects of the absorption of the products of growth when saprophytic or simple parasitic bacteria grow upon diseased or injured tissues.

A "focal infection" is one which acts as a focus for the dissemination of infectious material to other parts of the body. A focal infection may be the initial point of infection or may have been originally hematogenous in origin. An example would be an apical abscess of the tooth, which may later be a focus for infection of the endocardium, the joints, etc.

**Sources of Exogenous Infection.**—As has been stated pathogenic microörganisms are usually most virulent when in or just as they leave the body. As a general rule, therefore, the more direct the transfer from host to host the greater is the probability of infection. For the transfer of infection and the continuance of a disease, it is necessary that microorganisms be discharged from the body. The avenues of exit will depend upon the site of localization. In the case of localized infections they will be discharged in the secretions or excretions from this area (saliva, sputum, urine, feces, etc.) or in case of abscesses in the pus from the broken down lesion. In disseminated infections the microörganisms may be discharged to a variable extent in a number of the secretions or excretions.

Diseases which are directly transmissible are called "contagious." Upon the virulence, the amount of the virus excreted and relative and numerical susceptibility of those exposed will depend the degree of "contagiousness." Contagious diseases are less frequently transferred by contamination of inanimate objects, "fomites," such as eating utensils, clothing, bedding, contents of rooms, money, etc. The probability of transfer is in direct proportion to time elapsing since contamination of the "fomes." Immediate transfer is the most dangerous.

Food and drinking material may be contaminated and be a serious source of infection. The probability of infection is enhanced if the material serves as a foodstuff for the further development of the contaminating agent, as, for instance, streptococci and B. typhosus in milk.

Insects may act as a mechanical means for the transfer of disease. Biting insects may be the only mode by which the virus of the disease finds an exit from the infected host as in malaria. In most instances, especially in the case of protozoan diseases, a life cycle must be completed in this "intermediate host" before the virus becomes transferable to a new host. In some instances the virus is transmitted to the insect's offspring, thus in "tick fever" of cattle it is the offspring, not the biting female that transfers the disease.

Transfer may occur by a third person acting as a mechanical agent. This is apt to occur when cleansing of the exposed skin of the third person is omitted or inadequate and the hair and clothes are not protected from contamination. Where any considerable time elapses since contact, there is a strong probability that the transfer is due to the development of the "carrier state."

**Carriers of Disease Germs.**—An individual may become a carrier following exposure to infection. This carrier condition may develop without any evidence of actual disease. In this case the individual possesses an immunity to the disease, but the infectious agent becomes implanted as a simple parasite. Such a carrier is called a "normal or contact" carrier. Should disease develop in a susceptible person and recovery take place, the infectious agent may remain as a simple parasite, the body being protected from further injury due to the immunity developed in the process of recovery of the disease. Such a carrier is a "convalescent carrier." Frequently the "convalescent carrier" state is due to the persistence of chronic foci of inflammation in the crypts of the tonsils, in the gall-bladder, or elsewhere. Because of the situation of these foci and the character of the local secretions the infectious agent is protected from antibody activity. These lesions remain limited and the body is protected from reinvasion or reinfection, due to the protective mechanism which developed and caused recovery from the disease.

Carriers, both normal and convalescent, may be transient, temporary or chronic. The last is more likely to occur in convalescent carriers. Contact carriers although usually transient or temporary may become chronic. There is reason to believe that the infectious agent, B. typhosus, for instance, may invade the body, however, failing to develop because of the host's immunity, and produce the same type of chronic localized lesion in the biliary system, as in a case of disease.

The carrier, therefore, is in some respects a more dangerous source of infection than is the diseased individual. He not only excretes the infectious agent, but may also because of his healthy condition be unsuspected. Equally insidious sources of infection are cases of disease with mild or negligible symptoms, the so-called "missed" or "passed" cases.

**Local Effects of Infection.**—Microörganisms in sufficient numbers, even though they possessed no toxic qualities, would cause a local interference, as would other foreign bodies in the tissues. Possessed as they are, however, of toxic substances the latter is the dominant cause of local response. Various kinds of inflammation may develop, such as serous, fibrinous, hemorrhagic, catarrhal, croupous, membranous, purulent, necrotic, gangrenous or proliferative or productive. An organism may incite an inflammation that is more or less specific, in that one of these types of inflammation dominates. The same type of inflammation, however, may be caused by other types. Some produce no peculiar response. The character of the inflammatory response depends to a great extent on the site of the lesion, and depending on the site of localization, may differ considerably even with one and the same microörganism. The character and extent of the local lesion will depend on the virulence, the number introduced and the local resistance of the tissues. The following are examples of the types of inflammatory response to infection by various microörganisms. Thus inflammation and serous exudation into the subcutaneous tissues follow injections of the pneumococcus or anthrax bacillus. The development of the streptococcus or pneumococcus in the endocardium or pleural cavity is followed by a serous exudation, frequently with more or less fibrin production. The formation of pus results more especially from the streptococcus, pneumococcus and staphylococcus; but nearly all forms of bacteria, when they accumulate in one locality may produce purulent inflammation. The colon, typhoid, and influenza bacilli frequently cause the formation of abscesses.

Catarrhal inflammation, with or without pus, follows the absorption of products of many bacteria, such as the gonococcus, pneumococcus, streptococcus and influenza bacillus, etc. The hemorrhagic exudation seen in pneumonia is usually due to the pneumococcus; it is observed also in other infections. Cell necrosis is produced frequently by the products of the diphtheria and of the typhoid bacilli and by those of other bacteria. Specific proliferative inflammation follows the localization of the products derived from the tubercle bacillus and the leprosy bacillus.

**General Effects of Infection.**—The systemic effects of infection will depend to some extend upon the localization or dissemination of the infection, that is, upon the disturbance of the structure and physiological function of an organ which in turn may influence the functions of one or more other organs. Such functional disturbance will depend as well upon the selective affinity of the toxin elaborated by the infecting agent.

Thus the effects on the lung of pneumococcus infection are different from those of an infection of the meninges or of a pneumococcus septicemia, although there is basically the same type of intoxication with pneumococcus products. In the case of pneumonia the most serious symptoms may not be due to the lung involvement but to the heart through mechanical interference with the circulation as well as the toxic effects of the products of infection on the heart muscle.

There is one general effect common to all infections, where sufficient bacterial products find their way to the blood and lymph, viz., fever. This may be directly due to toxins secreted by the bacteria or indirectly to the absorption of and presumably to the digestion products of the bacterial protein. (See below Toxins and Endotoxins.) As a rule the degree of fever is an index to the degree of systemic toxemia. When, however, there is an overwhelming infection or when the body cells are exhausted, little febrile response may occur or the temperature may be subnormal.

**The Causation of Local and General Effects of Infection.**—Although microörganisms are foreign bodies, we cannot account for the disturbance they create on the basis of mechanical injury alone. To some extent, obstruction of the capillaries by the microörganisms or pressure on the capillaries as well as upon the lymphatics and tissue cells which incident to inflammation contribute to tissue injury or destruction. Even in the extreme types of fatal septicemia such as anthrax, such factors, however, give little basis for explaining the clinical and pathological manifestations.

One of the earliest attempts to explain the toxemic effects of infection was the hypothesis that non-specific poisonous products were produced when microörganisms utilized the proteins of the host for food. Some of the cleavage products of proteins, "ptomains," formed by bacterial metabolism, were shown to be toxic. These substances are essentially putrefactive products, being the intermediate products of proteolysis, such as putrescin, cadavarin or cleavage of lipoids, such as the lecithin derivatives neurin and muscarin. That ptomains have any appreciable role in producing the effects of infection is very doubtful. They are produced by many bacteria which are wholly without pathogenic power, whereas highly pathogenic types commonly produce little or no ptomains. Even when ptomains are produced by a pathogenic bacterium the effect of their injection is physiologically very different from that of the toxic products produced in infection.

The role of the ptomains in the production of disease is probably limited to the gastro-intestinal symptoms caused by spoiled foods. Possibly certain forms of intestinal "auto-intoxication" may be due to the production of ptomains in the intestinal canal. Ptomain production and absorption may be a factor in the mixed infections of extensive areas of dead tissue as in gangrene, crushed or lacerated wounds and deep burns.

It was soon evident to earlier investigators that common poisonous substances would not explain the more or less specific effects produced by different pathogenic microörganisms. Considerable doubt has arisen as to whether the purified ptomains as isolated by the chemist are the actual poisoning products of bacterial growth and tissue necrosis or are other cleavage products resulting from chemical manipulation.

Investigation, therefore, turned in the direction of the demonstration not of general toxic substances but of specific toxic substances. Roux and Yersin, in 1889, discovered that the diphtheria bacillus when grown in broth secreted and excreted a toxin which was present in the broth even though the bacilli were removed by filtration. The poison, therefore, was "extracellular" and "soluble." In 1891 Kitasato discovered a soluble toxin in the filtrates of broth cultures of the tetanus bacillus. Both these toxins were found to reproduce the physiological effects noted in infection by their respective types of bacilli.

With these discoveries it seemed that but a short time would elapse before the demonstration of similar toxins for all pathogenic microorganisms. It was soon found, however, that most bacteria, for example the typhoid bacillus or the cholera vibrio, did not produce demonstrable extracellular toxins, but that the toxic substances were bound up with the bacterial protoplasm during life and were only released after death and disintegration of the bacterial cell. Pfeiffer, in 1892, published such results and called these toxins "endotoxins" in contrast to the "exotoxins," above described. At first it was thought these "endotoxins" were as specific and definite in character as the exotoxins. Further investigations have shown that true "endotoxins," that is, endocellular substance with direct toxic capacities, probably do not exist. Various hypotheses have been advanced to explain the evident toxic effects of the endocellular substances of microörganisms. All assume that the toxic effects are indirect. The prevailing theory is that the toxic effects are due to intermediate cleavage products of the bacterial protoplasm. Vaughan's theories have received wide acceptance. His statement at a recent lecture is as follows:

> "(*a*) The infective bacteria, taking the colon, typhoid, tubercle, and the pneumococcus as types, contain an intracellular poison. This is not a toxin; it is not specific; it produces no antibody when injected into animals. (*b*) These bacteria elaborate no soluble toxin or poison. In old cultures there may be a trace of poison, but this results from the autolysis of the cells and is not a cellular secretion. (*c*) This poison is a group in the protein molecule and can be obtained in soluble form only after cleavage of the cellular proteins, which may be accomplished by superheated steam, dilute acids, or alkalis. (*d*) It exists in all true proteins, in pathogenic and non-pathogenic bacteria, and in vegetable and animal proteins. (*e*) It may result from the cleavage action of proteolytic ferments. (*f*) In most vegetable and animal proteins the poisonous group is neutralized by combination with non-poisonous groups; consequently such proteins have no poisonous action until they undergo molecular disruption. (*g*) This primary group is poisonous because of the avidity with which it combines with secondary groups in the proteins of the animal body. (*h*) The specificity of proteins lies in their secondary, non-poisonous groups. It is in these that one protein chemically and biologically differs from another. (*i*) Biologic relationship among proteins is determined by the chemical structure of their molecules. There are as many kinds of proteins as there are kinds of cells. (*k*) The symptoms of the infections differ chiefly on account of the organ or tissue in which the virus accumulates and where it is split up and its poison liberated. (*l*) The ferment which causes the cleavage of the bacterial proteins in the different infectious diseases is specific.

This theory as well as others will be discussed in detail in relation to the anaphylaxis.

**The Specific Responses of the Body.**—When the filtrate of a seven-day broth culture of the diphtheria bacillus is injected into animals in sublethal doses, for instance the horse, the cells react with the production of an antibody against the toxin. This substance is called "antitoxin." Its production is shown by the fact that increasing doses of the toxin can be injected until hundreds or thousands of what originally would have been fatal doses are given. Likewise, it can be shown that mixing the blood or serum of the immunized horse with toxin will result in neutralization of the toxin. This neutralization follows roughly a multiple scale, thus if one part of serum will neutralize one part of toxin, one thousand parts of serum will approximately neutralize one thousand parts of toxin.

If the filtrate of a seven-day broth culture of the typhoid be injected, the antibodies produced are practically all antibacterial in character. The filtrate may be toxic, and we find that the size of successive doses can be increased, but not to any extent comparable with that noted above. After a series of injections, mixing the blood or serum of the horse mixed with the filtrate, will neutralize the toxic action of the filtrate to some extent, but such neutralization will not follow a multiple scale. As the amount of "endotoxin" is increased the multiple of serum required will have to be much greater. If the amount of "endotoxin" is still further increased, neutralization becomes difficult or impossible, however much serum is added. If further tests are carried out it will be found that the antibodies produced are dominately antibacterial in character: that is, the antibodies are antiprotein in character, the bacillus substance stimulating their production.

The obvious deductions, therefore, would be that the response of the body in the attempt to recover from an infection would differ very materially as to whether the infecting organism produced or did not produce an extracellular toxin. In the absence of such a toxin the response should be primarily in the production of antibacterial antibodies, which observation and study has shown to be the case.

In infections by microörganisms which produce their effect by means of extracellular toxins, for example the diphtheria bacillus, the body response should be primarily the production of an antitoxin, which produced in sufficient amounts should neutralize the toxins present and the toxin as it is produced, so that the neutralized bacillus could be destroyed by the action of antibacterial antibodies and cells. As a matter of fact, this is not the essential mechanism of recovery, as is shown by the fact that about 75 per cent. of individuals remain relatively susceptible to diphtheria toxin after recovery: that is, they have produced antibacterial antibodies, but no antitoxin. In spite of this, as we shall see, we can stimulate the production of antitoxin in such individuals, that is, cause them to develop an antitoxic immunity which will be complete and probably permanent.

The mechanism underlying recovery therefore in all infections is essentially antibacterial in character: that is, by the development of specific antibacterial antibodies and their action as well as the activity of the phagocytic cells.

Another phenomenon is intimately associated with the process of recovery as well as with the development of immunity, viz., hypersensitiveness or anaphylaxis. (See later chapter.) No attempt is made at this place to discuss the apparent neutralization of the "endotoxic" types of bacterial poisons, as this also involves a survey of the theories of protein hypersensitiveness.

There is no demonstrable difference in the phagocytic cells themselves before and after the acquisition of active immunity: that is, an acquired increased phagocytic power of the cells does not play any part in active immunity. There may be present, however, an increased number of those antibodies which act as an aid to phagocytosis.

SUMMARY.—Natural resistance to invasion by bacteria is due to some extent to the protective coverings of the physiological exterior of the body as well as to the protective action of the secretions which are discharged on these surfaces. Natural resistance or immunity to infection may exist because of the possession of antibodies as well as because of the activities of the phagocytic cells. A natural immunity may be specific, in which case the immunity is usually antibacterial in character. Such an immunity is only relative. In the case of diphtheria a natural antitoxic immunity may exist which is complete. Infection causes a more or less marked increase is antibacterial antibodies which are apparently essential in the recovery from disease. Phagocytosis likewise is an important factor in such recovery. An acquired immunity, through infection or artificial inoculation by microörganisms which do not produce an extracellular toxin, is probably also dependent upon the stimulus to the development of specific antibacterial antibodies. In both the mechanism of recovery from disease and of acquired immunity, sensitization of the cells to bacterial products is an important factor. An acquired antitoxic immunity develops after infection in only a small proportion of cases. It can be produced in nearly all susceptible persons through inoculation.

*Theories Concerning the Production and Action of Antibodies.*—It was soon evident to early investigators especially in the light of the large amounts of antibodies that might be produced in response to the injection of small amounts of toxin, that the antibodies were secreted by the body cells and were not due to a conversion of bacterial products.

On this basis Ehrlich elaborated his well-known "side-chain theory." This theory was first advanced in the attempt to explain the nutritive process of cells. He conceived the cell as consisting of a complex central chemical nucleus of relatively constant structure to which the cell owed its peculiar functional character. Attached to this are many "side chains" which because of their affinities can take the appropriate food molecules from the body fluids. After such combination the food molecule is assimilated and incorporated in the central nucleus. This conception places the nutritive activities on a chemical basis. When infection occurs the toxins or other products combine with the cell by the same mechanism if there is an affinity between any of the side chains and the molecules of toxin or other microbal product. These products may find suitable side-chain, "receptors or haptines," in many varieties of cells or in only the cells of certain tissues or organs. When the microbal products combine with the side chains the molecules are available not simply as foodstuffs but are actually deleterious and the side chain or receptor is destroyed; or if sufficient receptors are involved the central nucleus is injured and dies. When the nucleus lives the receptors are regenerated. In this regeneration, however, the conception of overproduction as advanced by Weigert comes into play: that is, the injury not only stimulates the regeneration of the destroyed receptors but an excess of these receptors. The cell possessed of more recep-

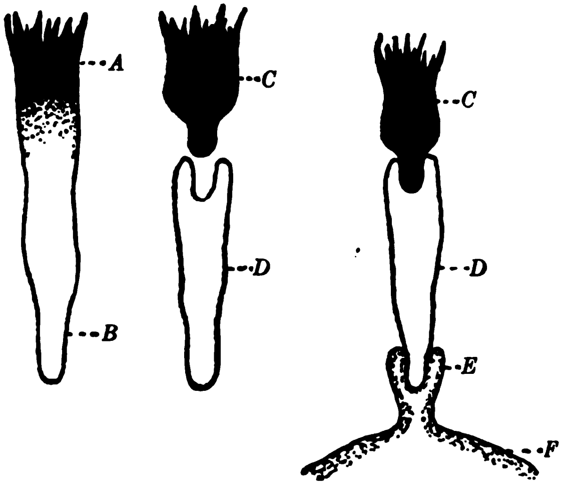

Fig. 72.—Graphic representation of receptors of the first and third orders and of complement as conceived by Ehrlich: *A*, toxophorous group of toxin; *B*, haptophorous group; *C*, complement; *D*, intermediary or immune body; *E*, foreign cell receptor; *F*, part of cell. *A B* unites directly with *E*; *C* unites by means of *D*.

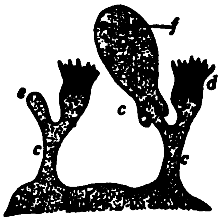

Fig. 73.—Receptors of the second order. Here *e* represents the haptophore group, and *d* the zymophore group of the receptor, *f* being the food molecule with which this receptor combines. Such receptors are possessed by agglutinins and precipitins. It is to be noted that the zymophore group is an integral part of the receptor.

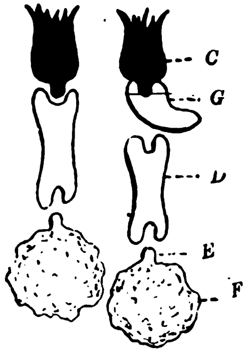

Fig. 74.—Graphic representation of amboceptors or receptors of the third order and of complement, showing on left the immune body uniting complement to foreign cell and on right the action of anti-complement, binding complement and so preventing its union with the ambocepter (see Fig. 67). *C*, complement; *D*, immune body; *E*, receptor; *F*, foreign cell; *G*, anti-complement.

tors than necessary for its nutritive process casts the excess off into the body fluids, these cast off receptors now constituting free antibodies. It is conceivable that if the stimuli to the cells were continued by the repeated partial destruction of receptors, that large amounts of antibodies would be formed in this way. The theory explains the general specific character of the antibodies as only those cell receptors are overproduced which have an affinity for the products of the infecting organism. The very bodies that when connected to the cell "sessile" made possible the poisoning of the cell, constitute the antibodies when free.

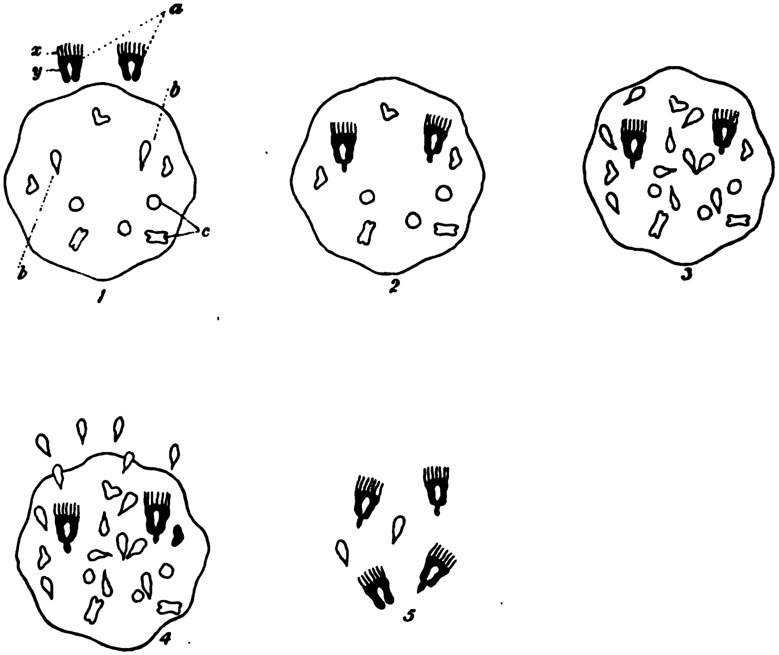

Fig. 75.—Graphic representation of Ehrlich's theory of the production of antitoxin and the neutralization of toxin: *a*, diphtheria toxin molecule; *x*, toxophore atom group; *y*, haptophore or combining group; *b*, cell receptors with affinity for diphtheria toxin; *c*, other cell receptors. 1, cell with its receptors—outside of cell, free toxin molecules; 2, toxin molecules combined with the cell receptors having affinity for diphtheria toxin; 3, after three days, showing multiplication of cell receptors similar to those combined with toxin others not increased; 4, after four days, excess of toxin receptors cast off in the blood; 5, toxin molecules being neutralized by combining with free receptors in blood into which antitoxin had been transferred.

These antibodies, therefore, having an affinity for microbal products will chemically combine with and neutralize them.

This theory was applied to the origin and action of the simpler antibody, antitoxin and then elaborated to include the more complex antibacterial antibodies. Graphic representations of the theoretical structure, affinities and mode of action, according to Ehrlich, of the "antigens" (stimuli to antibody production) and antibodies are given below. The different types of antibodies and the validity of these conceptions in tion to each kind of antibody are taken up in the following chapters.

# CHAPTER VIII.

## TOXIN AND ANTITOXIN.

As has been stated, microbal antigens, that is, the substances stimulating the production of microbal antibodies, can be divided into two classes: (1) the extracellular products, among which we have the exotoxins, and (2) the endocellular products or microbal proteins. The first stimulate the production of antitoxins, the second stimulate the production of antimicrobal or antiprotein antibodies, viz., agglutinins, precipitins, bacteriolytic, bactericidal or other microbicidal substances and opsonins. Each of these varieties of antibodies, as we shall see, can be produced by the injection of other substances than microörganisms or their products.

**Toxins.**—The extracellular soluble toxins, exotoxins, are produced by only a limited number of microörganisms. The chief examples are diphtheria, tetanus and botulism toxins and that of the anaërobes producing gas gangrene. The essential characteristics of exotoxins, are (1) their great lability, (2) their production of symptoms of poisoning, however large the dose, only after a period of incubation, (3) in appropriate doses, their stimulation of the production of an antitoxin which has the capacity of neutralizing the toxin.

**Other Characteristics of Exotoxins.**—Chemically they resemble toxalbumin, albumoses or are at least allied to the albumoses. They are soluble, non-crystallizable and do not dialyze except through very thin membranes. They are digested by proteolytic ferments. Their marked lability makes them sensitive to light, free oxygen or oxidizing agents and to heat, diphtheria and tetanus toxin being destroyed by heating to 60° C. for one-half hour; even heating 56° C. for one-half hour destroys 95 per cent.

They are precipitated by ammonium sulphate, alcohol and nucleic acid. Although considerable deterioration may occur during the process of precipitation, especially with tetanus toxin, the dried precipitate stored *in vacuo* at low temperatures deteriorates very little.

**Methods of Precipitating Exotoxins.**—Ammonium sulphate crystals are added to the fluid containing the toxin until it is saturated. A large excess of ammonium sulphate crystals is then added and the whole kept at about 37° C. for twelve to eighteen hours. The toxin is thrown out of solution along with the albumoses and peptones and rises to the surface. This is skimmed off and dried in a vacuum or in an exsiccator containing strong sulphuric acid. The dried powder is placed in vacuum tubes and stored in the dark. Banzhaf has obtained a diphtheria toxin that is practically free from the meat extractives in the broth. He adds alcohol up to 65 per cent. to the slightly acidified toxin broth. The small flocculent precipitate that is formed, after standing about one hour, is filtered off and dried in vacuum. This dried toxin does not deteriorate. One gram of the powder contains 40,000 fatal doses, whereas the original toxin broth contained 5000 fatal doses per gram of solids.

**Nature and Structure of Toxins.**—They are synthetic products of bacterial growth and are specific. Ehrlich believed that the toxin molecule is composed of two essential parts: a haptophore portion which has an affinity for the cell receptor, and a toxophore group which caused the toxic effects (see Chapter VII). He believed that the deterioration of toxins, under ordinary conditions of storage, was primarily due to the deterioration of the more labile toxophore portion. Evidence has been advanced that the same type of deterioration is produced when the toxic action is destroyed by adding iodin or carbon bisulphide. Ehrlich called this residual haptophore portion a "toxoid." Theoretically, therefore, the toxoid should retain its ability to combine with cell receptors, but being no longer toxic or capable of causing irritation there should be no response in antitoxin production. To explain certain observations in standardizing toxin and antitoxin (see below), Ehrlich elaborated this theory by assuming that subvarieties of toxin and toxoids existed as manifested by differences in avidity for the cell receptor or for antitoxin. To explain the late paralysis of diphtheria toxemia he assumed the existence of a special toxic fraction which he called toxon. Late paralysis may develop in guinea-pigs in spite of antitoxin sufficient to prevent acute toxemia and death. Ehrlich assumed therefore a low affinity between toxon and antitoxin. If the amount of antitoxin is sufficiently large paralysis does not develop.

This conception implies a purely chemical nature in toxin. This seems to be sustained when one considers that the amount of injury done by toxin is comparable to the amount injected. On the other hand, the activity of infinitesimal doses in causing tissue injury or death or the disproportionate amount of antitoxin produced in response to toxin injection suggests strongly that the action of toxin is not purely chemical but more allied to the action of ferments. The fact that toxins cannot be obtained free of albuminous matter has been advanced as an added argument in favor of their enzyme character.

A broth culture of tetanus may be so toxic that 1 c.c. will suffice to kill 50,000 to 75,000 guinea-pigs. Yet the major part of this amount of broth culture is water and meat extractives and peptone, so that the actual amount of toxin which will suffice to kill one pig is exceedingly minute. Knorr has shown that the injection of one unit of toxin into a horse already immunized against tetanus will stimulate the production of 100,000 units of antitoxin, which is most striking when one considers that there already existed in the body fluids many times the amount of antitoxin necessary to completely neutralize the toxin before it could reach the cells. If the toxin were neutralized in a purely chemical way in the body fluids by combining with the antitoxin it is difficult to conceive how it could then have stimulated the production of the amount of antitoxin noted.

Against the enzyme nature of toxin we have the facts, however, that toxin when introduced into the body, no matter how large the amount, quickly disappears from the body fluids due to combination with the cells for which it has an affinity. This combination is very firm and is dissociated, if at all, only with great difficulty, indicating a chemical union. In the case of tetanus toxin the lipoids of the nerve cells have a very marked influence on toxin-cell combination, indicating again that physical phenomena at least underlie the union. (See also under Combination of Toxin and Antitoxin.)

**Special Affinities of Exotoxins.**—It is probable that toxins when present in sufficient amounts have some effect on all cells. Different toxins, however, cause their major or dominant symptoms by selective action on certain tissues or organs. Thus diphtheria toxin affects the vascular nerve centers and causes degeneration in the heart muscles, liver and kidneys. A specific action on the suprarenals is regularly noticed in guinea-pigs. The glands are enlarged and congested and microscopically show a degeneration or disappearance of the chromatin substances and of the medullary cells.

Tetanus toxin, according to Ehrlich, is composed of two substances: tetanospasmin, which affects the nerve cells, and tetanolysin, destructive for red blood cells. The toxin, or at least the tetanospasmin, is apparently absorbed from the blood and tissues by the nerve-endings and finds its way to the central nervous system by way of the neuron. The affinity of tetanus toxin both for the nerve cells and for the red cells is apparently related to the lipoidal content of these cells.

The toxin of B. dysenteriæ (Shiga) affects the intestinal mucosa during natural infection. When the toxin is intravenously injected into animals a similar effect is produced.

Toxins may show a very definite affinity for a limited area of a tissue like the nervous system. Thus the more characteristic symptoms of botulism-poisoning are due to paralysis of the muscles supplied by the cranial nerves, those supplied by the third being first affected.

**Measurement of Toxin Action.**—This is done by determining the smallest dose which will kill a susceptible animal in a certain time. To obtain comparable results a certain mode of injection and the selection of a definite animal weight must be adhered to. This dose of toxin is called the "minimum lethal dose," or the M. L. D. The standard for diphtheria toxin is that amount of toxin which, on the average, will kill a guinea-pig weighing 250 grams on the termination of the fourth day. The standard for tetanus toxin is the amount that will kill a guinea-pig weighing 350 grams in the same time. Injections are made subcutaneously.

**Methods of Producing Exotoxins.**—See Specific Organisms.

**Antitoxin, Nature and Structure.**—An antitoxin is a specific antibody produced in response to an exotoxin and has the capacity of neutralizing only that exotoxin which stimulates its production.

Antitoxins have not been obtained in a pure state. When plasma or serum is fractionally precipitated, the antitoxin is precipitated with the globulin fraction. With diphtheria and tetanus antitoxin obtained from the horse all of the antitoxin precipitates with the pseudoglobulins.

This indicates that the antitoxin is a globulin or more likely that it is associated with the globulin molecules. Antitoxin is a cellular product and according to Ehrlich's hypothesis consists of the overproduced side chains or receptors of the cells irritated or poisoned by the toxin. He characterizes antitoxin as an "antibody of the first order" because of its relatively simple structure, being only a haptophore group which combines directly with the toxin molecule. (See Chapter VII.)

There is considerable evidence against this conception of simple quantitative combination which is discussed in relation to the combination of toxin and antitoxin. Antitoxins are relatively stable, deteriorating not more than 15 per cent. a year when refrigerated or 20 per cent. at room temperature. When dried and preserved *in vacuo* in the refrigerator practically no deterioration takes place over long periods of time. Heating to beyond 62° C. destroys antitoxin, the rapidity of destruction being greater the higher the temperature.

**The Combination of Toxin and Antitoxin.**—If Ehrlich's conceptions of a simple chemical combination were correct then toxin and antitoxin should combine in a directly multiple way: that is, if one part of antitoxin would neutralize one part of toxin then 1000 parts of antitoxin should neutralize 1000 parts of toxin, however the method of mixture. The combination is relatively but not absolutely multiple. If we determine the amount of antitoxin necessary to neutralize a certain amount of toxin and then again, use the same quantities but add the toxin to the antitoxin in fractions, allowing considerable time to elapse between additions, the mixture will be found to be toxic.

In determining the unit value of diphtheria antitoxin according to the original Ehrlich definition unexpected phenomena were encountered. The unit referred to is the amount of antitoxin necessary to neutralize 100 M. L. D.'s of toxin. Different persons testing the same antitoxin obtained very different results unless the same sample of toxin was employed. Even with the same sample discrepant results were obtained when any considerable time elapsed between tests. Thus a freshly prepared toxin gave a greater unit than an old toxin. Ehrlich investigated these facts by comparing the effects of the M. L. D., the Lo and the L + dose of different toxins. The Lo dose refers to the amount of toxin exactly neutralized by one unit of antitoxin. The L + dose refers to the amount of toxin which when mixed with one unit of antitoxin will still cause death of the guinea-pig on the fourth or fifth day. Theoretically, therefore, the Lo dose should equal 100 M. L. D.'s the L+ dose 101 M. L. D.'s. This he found not to be the case and the quantitative relationships of one to the other differed at times very widely according to the samples of toxin employed. A toxin might give a M. L. D. dose of 0.0025 c.c. An Lo dose of 0.125 c.c. and L+ dose of 0.25 which showed that the theoretical values were not obtained. He observed also that although the M. L. D. might increase greatly in volume, due to deterioration of toxin, the Lo value remained more constant. He studied the subject further with a method of partial absorption. With this method he obtained results of which the following figures are representative. In the first line no antitoxin was used but the test of the M. L. D. content of the L+ dose, toxin is inserted for comparison.

Antitoxin 0 unit + toxin L + dose = 85.0 M. L. D.
Antitoxin 0.25 unit + toxin L + dose = 60.0 M. L. D.
Antitoxin 0.80 unit + toxin L + dose = 10.0 M. L. D.
Antitoxin 0.90 unit + toxin L + dose = 3.5 M. L. D.
Antitoxin 1.00 unit + toxin L + dose = 1.0 M. L. D.

To explain these results and still retain his hypothesis of a simple quantitative combination, he predicated the production by B. diphtheriæ of two primary substances, a toxin and a toxon, both of which bind antitoxin, the latter having the weaker affinity. The toxins and probably the toxons deteriorate to secondary substances, toxoids, which again differ in their avidity for antitoxin. In the final analysis Ehrlich assumed, therefore, that two modifications of toxin existed and that the valency or combining value of a unit was 200 not 100, or that one antitoxin unit represented 200 valencies; and when mixed with an L+ dose of toxin left one toxin unit free. Theoretically, therefore, if a toxin could be produced containing only toxic or killing units the Lo dose would contain 200 M. L. D.'s and the L+ dose 201 M. L. D. (See below Antitoxin Unit.) As Ehrlich assumes that toxin and toxon are produced, and as production is quickly followed by deterioration, even the earliest produced toxin would never approach the theoretical value given above. The highest value ever obtained (Madsen) was 160 M. L. D. Direct proof of Ehrlich's assumption is therefore impossible. The fact that Ehrlich was forced to assume the existence of many different kinds of toxin makes the whole hypothesis very doubtful.

Arrhenius and Madsen attempted to explain the phenomena noted above by comparing the combination of toxin and antitoxin to that which occurs with a weak acid and weak base. In this case the reaction would be incomplete and reversible, due to the hydrolytic action of the water. Chemical equilibrium would follow their mixture, but the combination would be incomplete, so that free base and acid would always be present. The proportions of these three constituents would depend on the concentrations of the solutions employed according to the physical law of mass action. Likewise, the fractional addition of one to the other would change the neutralizing value of the total amount added. Arrhenius and Madsen studied the values obtained by adding boric acid in fractional amounts to ammonia and found that the rate of neutralization was closely parallel to that obtained by Ehrlich in his partial absorption method.

It is likely that neither of these theories are valid. All that we know about toxin and antitoxin indicates that they are colloidal in nature. Bordet believes the combination of toxin and antitoxin to be an absorption phenomenon of two colloids. He believes that when antitoxin is added to toxin it is distributed equally over the toxic molecule. As more antitoxin is added there is a proportionate reduction in the toxicity of each molecule until complete neutralization results.

The reaction, therefore, is analogous to a staining operation, the dye staining all the substance to which it is added, the depth of the color being proportionate to the amount of dye added. There is much to commend in these views of Bordet.

**Standardization of Antitoxin.**—The classical definition of an antitoxin unit of diphtheria antitoxin is given above. Because of the phenomena already described this unit was modified by Ehrlich, so that a unit today refers to that amount of antitoxin which when added to an L+ dose of toxin will cause on injection the death of a 250-gram guinea-pig on the fourth or fifth day. Evidently the starting point of such a unit is a standard antitoxin with which one can first determine the L+ dose of our test toxin. The antitoxin was selected as the initial standard because its deterioration could be prevented more easily than with the toxin. A sample of antitoxin was originally selected and the unit value of this sample made the official unit. Today the Hygienic Laboratory supplies such a standard antitoxin by which toxin is standardized for use in antitoxin testing. The following is a copy of one of our tests for determining the strength of diphtheria toxin:

**Method of Standardizing Diphtheria Antitoxin.**—The L+ of the standard diphtheria toxin of any laboratory is determined by combining one unit of diphtheria antitoxin, as supplied by the Hygienic Laboratory, Washington, D. C., with an amount of diphtheria toxin that will kill a 250 gm. guinea-pig in three days.

| At present our L+ = 0.71 c.c. | 24 hrs. | 48 hrs. | 72 hrs. |
|---|---|---|---|
| 1 unit + 0.71 c.c. in G. P. 255 gm. | 235 | 220 | Dead. |
| 1 unit + 0.72 c.c. G. P. 250 gm. | 225 | Dead. | |

**Testing of Diphtheria Antitoxin.**—Example: 1300 units per cubic centimeter. 1 c.c. of antitoxin into 100 c.c. volumetric flask; saline up to 100 c.c.; dilution = $\frac{1}{100}$.

1 c.c. of above into a small flask + 12 c.c. saline; dilution 1 c.c. = $\frac{1}{1300}$.

1 c.c. of last dilution (1 unit) + 0.71 c.c. (L+) of standard toxin into another flask—allow to stand one-quarter hour—inject subcutaneously into a 250 gm. guinea-pig.

Death at end of three full days—after daily loss of weight and varying degrees of induration.

| | 24 hrs. | 48 hrs. | 72 hrs. | |
|---|---|---|---|---|
| Anti. prep. $\frac{1}{1300}$ + 0.71 c.c. G. P. 250 gm. | 235 | 220 | 210 | Dead P.M. |
| | sl. Id. | Id. | Id. | |

Autopsy—congestion at site of injection and adrenals.

We prefer to have our guinea-pigs live five or six days, so that what we label a unit is really slightly more.

In the case of tetanus it was found that precipitated and dried toxin could be kept constant and for testing purposes this is being sent out by central controlling laboratories such as the Hygienic Laboratory at Washington instead of the standard antitoxin. A unit may be defined as that amount of antitoxin which will protect a 350-gram guinea-pig from 1000 fatal doses of standard toxin.

*Intracutaneous Method.*—Amounts of toxin too small to kill a gineau-pig can be demonstrated by intracutaneous injection, as evidenced by the development of edema and a small area of necrosis. Roemer utilized this fact for a method of standardization. This method is relatively accurate in experienced hands. It has an advantage of economy of pigs as several tests can be done with one pig, and is especially applicable in testing for small amounts of antitoxin. The starting-point is to determine the necrosis dose of toxin when mixed with a definite small fraction of a unit of antitoxin. A practical application of the intracutaneous method is the Schick reaction for the determination of virulence. (See Chapter XX.)

**Use of Toxin Neutralization in the Identification of Bacteria.**—As we shall see, immune reactions are commonly employed in the final identification of suspected pathogenic organisms. The determination of the morphologic and cultural reactions do not suffice, because for every type of pathogenic bacterium there are similar non-pathogenic types which may often be encountered in the same area or in the same excretions which are to be examined for the presence of a pathogenic variety.

If we isolate a bacterium which has the morphological and cultural reactions of an exotoxin-producing variety, for example a suspected diphtheria bacillus, we can prove it to be a diphtheria bacillus by demonstrating that it produces a toxin which is neutralized by known diphtheria antitoxin. (See Chapter XX.)

**Natural and Active Antitoxic Immunity.**—Antitoxic immunity may be produced by the injection of sublethal doses of toxin as well as by the injection of neutral or partially toxic mixtures of toxin and antitoxin. The latter method is utilized successfully for the immunization of man where an antitoxic immunity is absent. (See Chapter XX.)

The ease of production of antitoxin varies with different species and seems to be roughly proportionate to the percentage occurrence of natural antitoxin immunity in the species. Thus a natural antitoxic immunity is never encountered among guinea-pigs and they produce antitoxin with difficulty. An antitoxin immunity does not develop until six to eight weeks. The horse, on the other hand, commonly shows a natural antitoxic immunity, and as a species produces antitoxin with great ease. If, however, an individual horse possesses no initial antitoxin he again will produce antitoxin less easily. Man shows the characteristics of both the above species. Individuals possessing no natural antitoxin produce antitoxin in appreciable amounts only after the lapse of four to twelve weeks, whereas individuals possessing antitoxin respond promptly and freely in the production of more antitoxin. This has great practical importance in the active immunization of children against diphtheria. (See Chapter XX.)

**Passive Antitoxic Immunity.**—This can be conferred by the injection of preformed antitoxin. Antitoxin can be employed as a prophylactic measure for the prevention of diphtheria and tetanus; it may also be used as a therapeutic measure after the disease has developed. It must be clearly understood, however, that the therapeutic use of the antitoxin is really as a preventive measure, that is, it protects the cells from further toxic action and is of no value in restoring the cells already injured. Therefore, one can see the necessity of the earliest possible application of the antitoxin. A discussion of the practical use, absorption, mode of application, etc., is given in Part III.

**Production of Antitoxin for Therapeutic Use.**—**Diphtheria Antitoxin.**—**As a result of the work of years in the laboratories of the Health Department of New York City, the following may be laid down as a practical method:**

A strong diphtheria or tetanus toxin should be obtained by taking a very virulent culture and growing it in broth under the conditions described (p. 131). The following directions apply to the production of diphtheria antitoxin.

The horses used should be moderately young, vigorous, of fair size and absolutely healthy. The horses are severally injected with a preliminary injection of 3000 units of antitoxin. This gives a preliminary passive immunity and permits the injections of larger doses of toxin than would be otherwise allowable. The antitoxin does not interfere with further antitoxin production. On the day after the antitoxin is given an initial dose of 10 m. l. d. of toxin diluted to 50 c.c. with physiological salt solution is injected. Subsequent injections are given every second day, increasing the amount of toxin for the first seven to eight injections by 100 per cent. The rate of increase is 75 per cent. for the next seven to eight injections and 50 per cent. for the next series. The rate of increase is then gradually lowered to 10 per cent., which increase is maintained until the maximum for the horse is reached. Test bleedings are taken from time to time. When the unit content is sufficiently high (300 to 600 units) large bleedings are begun. Then the producing horse is given two injections two days apart, and bled on the seventh day after the latter injection. The series of two injections is started again the next day. The rate of increase of the toxin dosage is controlled by the temperature reactions and general condition of the horse. An elevation of more than 3° F. is undesirable.

There is no way of judging which horses will produce the highest grades of antitoxin. Very roughly, those horses which are extremely sensitive and those which react hardly at all are the poorest, but even here there are exceptions. The only way, therefore, is to bleed the horses and test their serum at the end of six weeks or two months. If only high grade is wanted, all horses that give less than 150 units per cubic centimeter are discarded. At the end of three months the antitoxic serum of all the horses should be over 300 units, and in about 10 per cent. as much as 800 units in each cubic centimeter. Not more than 1 per cent. give about 1000 units, and none so far has given as much as 2000 units per cubic centimeter. The very best horses, if pushed to their limit, continue to furnish blood containing the maximum amount of antitoxin for several months, and then, in spite of increasing injections of toxin, begin to furnish blood of gradually decreasing strength. If the injections are never beyond a moderate size and an interval of three months' freedom from inoculations is given every nine months; the best horses furnish high-grade serum during their periods of treatment for from two to four years.

**Tetanus Antitoxin.**—This is produced exactly as in the case of diphtheria antitoxin except that one only increases each dose by fifty per cent. Good horses yield a serum containing 200 to 600 units per cubic centimeter. They continue to produce serum of great potency for long periods. The toxin has a less deleterious effect on horses than the diphtheria toxin.

**The Handling of the Antitoxic Serum or Plasma.**—For the collection and preservation of the antitoxin the blood is withdrawn from the jugular vein by means of a sharp-pointed cannula, which is plunged through the vein wall, a slit having been made in the skin. When serum is to be obtained the blood is carried by a sterile rubber tube into large Erlenmeyer flasks, held slanted, or into cylindrical jars, and allowed to clot. The serum is drawn off after four days by means of sterile glass and rubber tubing, and is stored in large flasks. When the globulins are to be separated the blood may be added directly to one-tenth of its volume of a 10 per cent. solution of sodium citrate. This prevents clotting of the blood. The heavy cells settle to the bottom and the clear plasma is withdrawn. Tricresol 0.3 per cent. is added as preservative. Serum can be utilized immediately after testing for unit content and sterility.

**Separation and Concentration of Antitoxin.**—Ordinary antitoxic serum contains serum globulins, albumins, nucleoproteins, cholesterin, lecithin, traces of bile pigments and salts, non-protein compounds, inorganic salts and water. Pick and Atkinson showed that the antitoxins were present in the globulin fraction and that during immunization the content in globulins increased. Gibson and Banzhaf then showed that the fraction of globulins insoluble in saturated salt solution carried no antitoxin: that is, the pseudoglobulins not the euglobulins carried the antitoxin. The original concentration method of Gibson and Banzhaf was therefore to precipitate out the globulins by 50 per cent. concentration of ammonium sulphate, dissolve out pseudoglobulins with saturated salt solution and dialyze to remove the salt. Banzhaf then showed that heating to 57° to 62° C. for one hour rendered some of the pseudoglobulins fraction insoluble in sodium chloride, thus he attained a greater purification. The methods of Atkinson and Gibson and Banzhaf were worked out in the Laboratories of the Health Department of New York City and Banzhaf's continued experiments have resulted in the adoption of the following procedure now generally in use.

To the citrated plasma, containing 0.3 per cent. tricresol, add an equal volume of water. To seven parts of this mixture add three parts of a saturated solution of ammonium sulphate (30 per cent. saturation). Heat at 57° to 62° C. for one hour, filter while hot. This gives two fractions, thus:

| A. PRECIPITATE. | B. FILTRATE. |
|---|---|
| (Contains euglobulins, some pseudoglobulins and fibrinogen). | (Contains pseudoglobulins and albumins). |
| The precipitate is washed with 30 per cent. saturated ammonium sulphate. | Add washings from fraction A. |
| Dissolve in water and add NaCl to saturation (dissolves out the pseudoglobulins). Filter. | Add sufficient saturated ammonium sulphate solution to raise concentration to 50 per cent. (precipitates the pseudoglobulins). Filter. |
| Add acetic acid (to precipitate pseudoglobulins). Filter. | Precipitate pressed between pads to remove excess ammonium sulphate solution. |
| Precipitate pressed to remove excess water and acid. | Place in dialyzing bag, dialyze to remove salts. |
| Place precipitate in dialyzing bag and dialyze to remove salts. | Add NaCl to 0.8 per cent. and preservative, tricresol 0.4 per cent. |
| Add sodium chloride 0.8 per cent. and preservative tricresol 0.4 per cent. | |

Filter fractions separately through paper pulp and Berkefeld filter (latter to remove bacteria) test fractions for unitage and sterility.

Both fractions may be mixed together. In practice, because the "A" fraction has a lower unitage per gram protein and also because it is finished later, the two are not mixed. The A fractions from successive preparations are usually accumulated and then mixed.

**Advantages of Concentration of Antitoxin.**—With the method just given the finished antitoxin has 4 to 6 times the unit content of the original plasma. The total proteins as compared with serum antitoxin having the same total units is reduced about one-half. The result is that a larger unit dose may be given with the introduction of less foreign protein. This has resulted in a considerable decrease in the serum reactions especially rashes. The heating also reduces the capacity of the proteins remaining to produce rashes and fever after injection.

**Toxins Other than those of Microbal Origin and their Antitoxins.**—Plants as well as animals secrete substances which have many or all of the characteristics of microbal exotoxins. They may be characterized as phytotoxin (plant origin) and zoötoxins (animal origin). Examples of the former are ricin from the seed of the castor oil plant (ricinus communis), crotin, from the seed of a tree (croton tiglii) and abrin from the seed of the Indian licorice (abrus precatorius). They are toxic and when injected, stimulate the production of an antitoxin. As they agglutinate or hemolyze red cells (see later), and because they are relatively stable, they have been utilized to study the laws of combination of toxin and antitoxin, by means of test-tube reactions. The zoötoxins are the venoms of snakes, spider, scorpion, etc. The snake venoms contain toxic elements active against nerve cells, red cells and endothelium. They differ from microbal exotoxins in being relatively heat resistant, withstanding heating up to 75° C.; cobra venom is not destroyed even by heating to 100° C. for a very short time. Antitoxins of moderate potency can be produced for each of these venoms. As has been noted there is some similarity between the action of toxins and of enzymes. Antienzymes can be produced by animal injections which are specific and will neutralize the enzyme injected.

# CHAPTER IX.

## ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.

### AGGLUTININS.

By the phenomenon of agglutination is meant the aggregation into clumps of microörganisms uniformly dispersed in a fluid. If the organisms are motile they become immobile during this process. Many substances other than those in serum cause the agglutination of cells. We are as bacteriologists interested in these chiefly because they may cause us to believe we have noted agglutination phenomena from serum antibodies when really they were due to non-specific chemical substances.

This phenomenon (agglutination), while it had been noted by earlier observers (Charrin and Roger in 1889), was first extensively studied by Gruber and Durham in 1896, who determined that the serum of those passing through certain infections contained a specific substance (agglutinin) which caused the infecting organisms to clump. Several months later Widal reported that in typhoid fever the development of agglutinins could be used for diagnostic purposes. It was thus demonstrated by these studies and those of Grunbaum, Bordet and others that through agglutinins a new means was available for the identification of bacteria and in many cases the nature of the infectious organism causing disease.

**Agglutinogen.**—The antigenic substance stimulating the production of agglutinins is called the agglutinogen. It is formed in the living or dead microbal cell and appears to be released in cultures only on the dissolution of the organism. Heat and chemicals tend to destroy or alter agglutinogenic action. According to the Ehrlich hypothesis we may look upon the agglutinogen as a protein molecule having a haptophore group through which it combines with the cell receptor. Evidence has been advanced that the agglutinogen is contained in the ectoplasmic portion of microörganisms.

**Nature and Structure of Agglutinins.**—Agglutinins resist heating to 60° C., but above this temperature they are gradually destroyed, the destruction being complete at 75° C. They are not dialyzable and are precipitated with the globulin fraction of the serum. They are most active in a neutral or slightly acid menstruum. Alkalis tend to inhibit their action. They are the products of cellular activity and according to Ehrlich are more complex than the antitoxins in that they possess both a haptophore group by which they combine with the receptor of the microbal cell, but also a ferment portion or zymophore group to which they owe their action. Because of this greater complexity Ehrlich termed the agglutinins "antibodies of the second order." As under certain circumstances, such as the application of heat, acids, etc., agglutinins apparently lose their clumping but retain their combining power, Ehrlich drew a parallel with toxin and toxoids and called these altered agglutinins, "agglutinoids," that is, the zymophore or active portion might be destroyed but the haptophore fraction retain its affinity. (See Chapter VII.) This conception of agglutinins, like that of toxins cannot be sustained in the light of later work (see below). Agglutinins are specific within certain limitations and the combination with agglutinogen is approximately quantitative. They deteriorate more rapidly in dilute solutions, less rapidly in the undiluted serum. If the serum is dried and stored *in vacuo* the agglutinins will persist for long periods of time.

**Mechanism of the Agglutination Reaction.**—Agglutination is not a vital phenomenon. The microörganisms play a purely passive role. The Ehrlich conception of the combination and action of agglutinins is referred to in the preceding paragraph. Various reasons have been assigned by earlier investigators to explain the clumping, for instance Gruber advanced the hypothesis that the agglutinins changed the surface of the bacteria so that they stuck together.

The observations of Bordet have shown such theories to be untenable. He showed very definitely that agglutination was a physical phenomenon and that the combination of antigen and antibody was one phase, and agglutination another. Thus if a suspension of bacteria and a sample of serum be dialyzed until all salts are removed their mixture will not result in agglutination, unless salt be added. Or if the dialyzed mixture be centrifuged to remove the bacteria, and the sedimented bacteria suspended in salt solution agglutination will promptly occur. If sufficient bacteria have been used the supernatant fluid is free of agglutinins as shown by the absence of agglutination when salt and fresh bacteria are added. In other words combination occurs in absence of salts but agglutination only in their presence or, otherwise stated, agglutination is a clumping of the bacterium-antibody complex by salt action. These observations led to a comparison of the agglutination phenomenon to the flocculation of one colloid by another and the flocculation of colloids by salts and acids (electrolytes).

There is basis for the conception that a bacterial suspension is analogous to a colloidal suspension, differing from the latter only in the greater size of the suspension particles. The particles carry a similar electric charge which repels them one from another. They have a high surface tension which strives to reduce the free surface by bringing the particles together. As long as these forces are balanced the suspension remains stable. A colloidal suspension may be precipitated by the addition of an electrolyte; thus an albuminous solution is precipitated by the addition of a strong acid, the hydrogen ions neutralizing the electric charge, and the surface tension then causing agglomeration of the colloidal particles. Likewise colloids may be precipitated by a colloid carrying an opposite electric charge, although an excess may cause resolution. The addition of a colloid in too small amounts to result in flocculation "sensitizes" or makes possible the flocculation by amounts of electrolytes too small to otherwise cause flocculation. Another factor that must be considered is that a third colloid may prevent the mutual precipitation of two colloids ("protective colloid"); thus if serum be added to mastic it is protected from precipitation by ferric hydroxide. It can be readily shown that bacterial suspensions are agglutinated by electrolytes, for instance the acid agglutination of Michaelis. He found that many varieties of bacteria were agglutinated by acid each in a more or less specific zone of hydrogen dissociation. It is a relatively common occurrence that bacteria will remain in suspension in distilled water, but will fall out when suspensions are attempted with NaCl solutions as a preliminary to agglutination tests. This is termed "spontaneous agglutination." Yet in most instances we can readily prepare an even suspension in NaCl solutions. Evidently there is a difference in resistance in these cases to the influence of the electrolyte added. We may conceive of the absence and presence of a protective colloid in the bacterial ectoplasm, which when present would prevent agglutination by the oppositely charged electrolyte. Neisser and Friedmann believe that when agglutinins are added to their homologous bacteria in the presence of NaCl, the agglutinin is absorbed and neutralizes this protective colloid, agglutination resulting from the action of the electrolyte. On the other hand the agglutinin when absorbed changes the electrical charge, which in itself must also be a factor.

Although the above indicates that the mechanism of serum agglutination is closely analogous to that of the flocculation of colloids, and although it renders very doubtful the conception of Ehrlich as to the direct action of the agglutinin, the analogy fails to touch the question of specificity.[1] There must be some unknown chemical phenomenon at the basis of agglutination, which would explain the specific combination of antigen and antibody.

**Measurement of Agglutinins or Titration of an Agglutinating Serum.**—The agglutinating power of a serum is measured by determining the highest dilutions in which it still exhibits this quality. The titer of the serum as commonly used implies the highest dilutions in which complete agglutination still occurs. The results may be stated in another way, the titer is the smallest amount of serum which will agglutinate an arbitrarily selected standard amount of culture. The titer will vary according to the three factors, density of suspension, temperature of incubation and time of incubation. The denser the suspension the more agglutinins will be required. Agglutination proceeds relatively more rapidly as the temperature is raised from 20° to 55° C. Within limits, time of incubation likewise influences the degree of reaction. The reaction may be observed under the microscope, "microscopic method," or by the eye using test tubes or slide "macroscopic methods" (see below, Technic of Agglutination Reactions).

**Pre-zone or Proagglutinoid Phenomenon.**—Not infrequently it is observed that low dilutions of serum cause poor or even no agglutination, whereas higher dilutions will cause better or even a complete reaction.

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[1] Physical Chemistry, J. C. Phillip, p. 218.

This phenomenon is explained by Ehrlich as due to the presence of agglutinoids which, having a greater affinity for the bacteria, combine and prevent the action of the agglutinins. This phenomenon may be due to presence of a protective colloid. With either of these hypotheses we should assume that the substances are present in insufficient amounts in the higher dilutions. Similar zones are noted in the flocculation of one colloid by another, when one or the other is in excess. An excess of agglutinins alone is not sufficient explanation, however, as the occurrence of pre-zone phenomena does not parallel the titre of different sera.

**Specific (Major), Group and Normal (Minor) Agglutinins.**—Allied species of bacteria have protein substances that are more or less similar (see Fig. 76). If these common substances stimulate the production of agglutinins, the relative amount of each agglutinin should be somewhat proportional to the amount of agglutinogenic protoplasm possessed by the infecting or injected microörganism. Thus a serum from a typhoid patient may agglutinate B. typhosus in dilutions of 1 to 160, B. paratyphosus, 1 to 20, B. coli, not at all. The specific or major agglutinins should be present in greatest amount because the greatest stimulus is given by the preponderance of the specific typhoid protein, the group, common or minor in proportionately less amounts. This parallelism is not always followed, thus the serum from a case of paratyphoid fever may agglutinate B. typhosus as well as or even better than the infecting type. Likewise, the serum of an animal injected with B. paratyphosus B may agglutinate a related but distinct bacterium, B. choleræ suis, as well as the homologous bacterium. Several factors influence the relative production of major and minor agglutinins, especially the duration of the agglutinogenic stimulation, that is, the duration of the infection or the number of injections in the case of artificial immunization. The following chart serves as an example. Evidently the longer the period the greater is the probability of the production of considerable group agglutinins.

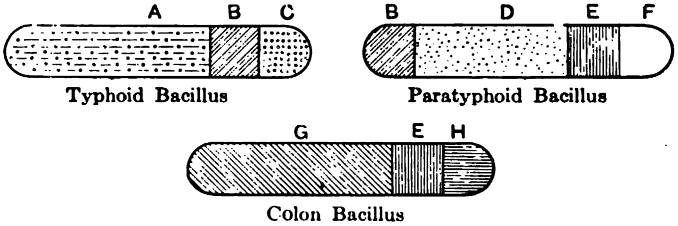

FIG. 76.—Specific and common agglutinins producing protoplasm.

Another factor is the nature of the host. Thus the rabbit, as a rule, produces less group agglutinins than does the goat, whereas the horse produces very large amounts of common agglutinins. There is a relationship between the production of group agglutinins and the degree to which agglutinins are normally possessed by the host. Normal agglutinins having group activity may be present for several or many varieties of bacteria. Thus the serum of man frequently agglutinates the typhoid or colon bacillus in dilutions of 1 to 1 or even 1 to 5. The serum from a normal horse may agglutinate these types in dilutions of 1 to 100 or 1 to 1000 or even more. The possession of such agglutinins we may assume to be due partly to the reaction to the continued absorption of intestinal bacteria of the colon group. Such an explanation, however, does not explain the fact that normal horse serum usually agglutinates the glanders bacillus in dilutions of 1 to 500 to 1 to 1000 or more, depending on the method used.

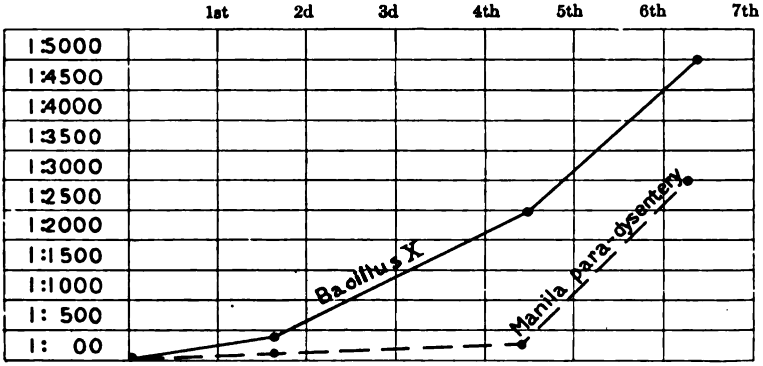

Fig. 77.—A young goat was used for the injection of the colon bacillus X. The great accumulation of common agglutinins for the paradysentery bacillus in the third month of the injections of the bacillus X is very striking.
● Tests made.

Possession of normal agglutinins in the species most commonly employed for the production of agglutinating serum is relatively as follows: rabbit, slight or absent; goat, moderate; horse, high to very high. This parallels, as above noted, the degree of group agglutinin production. If the varieties of normal agglutinins are carefully tested the paradoxical results obtained with some immune sera become clearer. Thus a horse serum may contain before injections a high content of agglutinins for two bacteria, B. dysenteriæ and B. coli. When injected the following results may be obtained.

AGGLUTININS IN THE SERUM OF A HORSE INJECTED WITH PARADYSENTERY BACILLUS, CULTURE TYPE MANILA.

| Culture. | After 18 injections. | | | After 21 injections. | | |
|---|---|---|---|---|---|---|
| | 1:3000 | 1:5000 | 1:10,000 | 1:3000 | 1:5000 | 1:10,000 |
| Paradysentery type Manila | ++ | – | – | ++ | ++ | ++ |
| Colon bacillus, type X | ++ | ++ | – | ++ | ++ | ++ |

Careful titration may show that such a horse had an excess of agglutinins for B. coli before immunization in which case the high "group" reaction is really the summation of group and normal agglutinins. This may not be the case, for equally paradoxical results may be obtained when the normal agglutinins for two related bacteria are slight in amount or even not demonstrable. This occurs at times even with injected rabbits. Why there should exist a greater sensitiveness to the group or common antigen we do not know. As we shall see (application and technic), reliable agglutination results are only possible when the above factors are carefully considered.

The above discussion assumes that the cultures employed are nearly equally agglutinable. Naturally, *apparently* similar paradoxes would occur if the heterologous strain was much the more agglutinable.

**Absorption of Agglutinins.**—This method advanced by Castellani allows us to differentiate with accuracy between specific and group agglutination. The specific agglutinins and also the common agglutinins which are stimulated by the infecting or injected bacterium should be bound by the homologous organism; in other words, if we add to a serum a large amount of its specific organism and then incubate we shall find that the clear serum obtained by centrifuge or filtration will no longer agglutinate either the specific organism or any of the related types previously agglutinated through the action of the group agglutinins. If we absorb similarly the serum with a related type we find the specific agglutinins will not be appreciably reduced, whereas the group agglutinins for the absorbing strain will have partly or wholly disappeared. Examples with moderate and extreme group agglutination are shown in the following tables.

ABSORPTION OF AGGLUTINATING SERUM OBTAINED BY INJECTION OF B. TYPHOSUS.

| | Highest dilution giving complete agglutination before absorption. | Absorbed by B. typhosus. | Absorbed by B. coli. |
|---|---|---|---|
| B. typhosus . . . | 1 to 5000 + + | 1 to 50 – | 1 to 4000 + + |
| B. coli . . . . | 1 to 1000 + + | 1 to 50 – | 1 to 50 – |

ABSORPTION OF AGGLUTINATING SERUM OBTAINED BY INJECTION OF B. PARATYPHOSUS B.

| | Agglutination before absorption. | Absorbed by B. paratyphosus "B." | Absorbed by B. choleræ suis. |
|---|---|---|---|
| B. paratyphosus B. | 1 to 10,000 + + | 1 to 50 – | 1 to 8000 + + |
| B. choleræ suis | 1 to 10,000 + + | 1 to 50 – | 1 to 50 – |

We have stated that the absorption by a heterologous bacterium will result in the absorption of the common or group agglutinins for itself. The absorbing type may absorb the common agglutinins for one or more other related types as well, thus:

ABSORPTION BY THE TYPHOID BACILLUS OF GROUP AGGLUTININS ACTING UPON A NUMBER OF VARIETIES OF B. COLI WHICH WERE STIMULATED BY ANOTHER VARIETY OF B. COLI. AGGLUTINATION BY SERUM OF RABBIT IMMUNIZED TO B. COLI X.

| | Before addition of typhoid bacilli. | After absorption with typhoid bacilli at 22° C. |
|---|---|---|
| Colon bacillus X . . . . . . . . | 600 | 5000 |
| Colon bacillus 1 . . . . . . . . | 500 | 20 |
| Colon bacillus 2 . . . . . . . . | 500 | 30 |
| Colon bacillus 3 . . . . . . . . | 250 | 30 |
| Colon bacillus 4 . . . . . . . . | 250 | 10 |
| Colon bacillus 5 . . . . . . . . | 10 | less than 10 |
| Colon bacillus 6–18 . . . | less than 10 | less than 10 |
| Typhoid bacillus . . . . | less than 10 | less than 10 |

An example of the results of absorption of agglutinating serum prepared by the injection of more than one variety is given in the following. Analogous results would occur in a mixed infection.

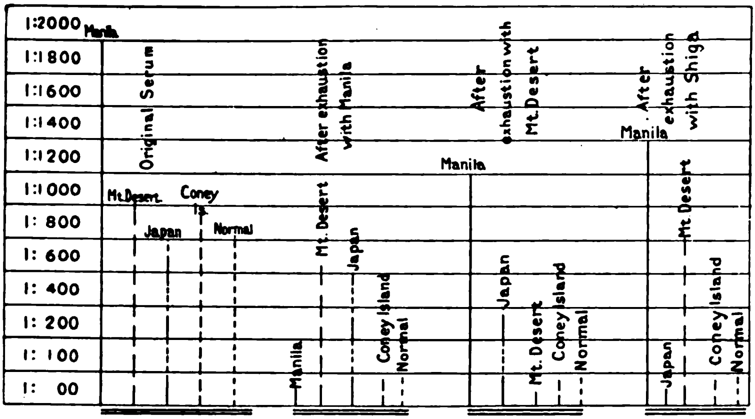

**Fig. 78.**—Showing the effect of saturating with bacilli of types of either Shiga, Manila, or Mt. Desert, a serum from a horse which had received combined injections of the three types. Note that the Manila type removed almost all the specific and group agglutinins acting upon its own type and the group agglutinins upon the Coney Island and normal types, leaving the specific agglutinins for types Shiga and Mt. Desert. The same is true for types Shiga and Mt. Desert when they were used.

Specific and group: ———————— Manila paradysentery. (Flexner.)
" " ———-----———--- Japan dysentery. (Shiga.)
" " — — — — — — — — Mt. Desert paradysentery or y. (Park-Hiss.)
Group only: - - - - - - - - - - - Atypical paradysentery. (Coney Island.)
" " ———————— Normal. (B. coli.)

Absorption by bacteria which are closely allied may reduce the agglutinins for each other as noted in the reduction of Manila (Flexner) agglutinins by the Mt. Desert strain given in the preceding chart. (See below under Technic of Absorption.)

One may obtain at times the following apparently contradictory results.

*Horse Serum.*—Horse immunized with B. typhosus.

| | Agglutination before absorption. | Absorbed by B. typhosus. | Absorbed by B. coli (m) |
|---|---|---|---|
| B. typhosus . . | 1 to 10,000++ | 1 to 50− | 1 to 8000++ |
| B. coli (n) . . . | 1 to 1,000++ | 1 to 50− | 1 to 50− |
| B. coli (m) . . . | 1 to 1,500++ | 1 to 300+ | 1 to 50− |

Why did B. typhosus not remove all the group agglutinins for B. coli (M)? The explanation is that the agglutinins remaining are normal agglutinins for which the homologous type has no absorbing protoplasm. Their normal presence was due to the stimulation by protein unrelated to that contained in the typhoid bacillus.

This is shown by testing the serum before immunization was started thus:

| | Agglutination before absorption. | Absorbed by B. typhosus. | Absorbed by B. coli (m). |
|---|---|---|---|
| B. typhosus . . . . | 1 to 500++ | 1 to 25− | 1 to 25− |
| B. coli (n) . . . . | 1 to 600++ | 1 to 25− | 1 to 25− |
| B. coli (m) . . . . | 1 to 500++ | 1 to 250++ | 1 to 25− |

Such unrelated normal agglutinins may increase during immunization and, because of a little understood effect of foreign protein, lead to serious errors both in agglutination and agglutinin absorption results if not scrupulously controlled.

Summarizing the above discussion, we may state that the absorption of a serum by its homologous organism will remove all the specific and group agglutinins produced in response to the proteins of that organism.

Therefore, if another bacterium gives the same absorptive result it is identified as the same type. Thus, all B. typhosus strains have the same ability to absorb the agglutinins from any antityphoid serum.

The ability of a type to absorb the group agglutinins for itself and one or more other bacteria can be taken only as an indication of some similarity in their protoplasmic content. It does not indicate necessarily any other relationship.

Absorptive results are susceptible to serious misinterpretation if character of the serum before immunization, especially the content in normal agglutinins be not known.

*Absorption results are of value only when we possess, or know, the specific type which caused the infection or was injected.* Thus the fact that B. typhosus removes all the agglutinins from a patient's serum both for B. typhosus and B. paratyphosus, whereas the latter only removes them for itself, is no guarantee that B. typhosus is the infecting bacterium. The infection may be due to a third related but unknown variety, the absorption results being group agglutinin absorption. Likewise, given a serum from which B. typhosus and B. paratyphosus will only completely remove the agglutinins for themselves is no certain proof of the existence of a mixed infection. The infection again may be due to a third unknown bacterium, the absorption resulting only in the removal of individual varieties of group agglutinins.

**Variations or Modification of Agglutinability.**—As a rule bacteria are relatively resistant to agglutination when freshly obtained from the body, especially if isolated from the blood or tissues. When cultivated on artificial media they become more sensitive to agglutination, and after a shorter or longer period many varieties tend to agglutinate spontaneously when grown in broth or suspended in salt solution. At times, organisms from the blood stream may be practically inagglutinable, becoming more agglutinable after cultivation for some time. The agglutinability of a culture can be varied; the conditions, as a rule, which tend to raise its virulence also make it less agglutinable (growth on blood immune serum, animal passage, etc.). As we shall see, these same means make a bacterium less susceptible to the action of other antibacterial antibodies. This suggests that the process is one of adaptation and possibly the production of anti-substances which neutralize or prevent the combination of the antibodies of the host. (See below.) The agglutinogenic power or power to stimulate the production of other antibodies is not lowered when bacteria become less agglutinable. The presence of a capsule may interfere with or prevent agglutination. The capsule, developing best in body fluids or tissues, is probably a protective substance. Porges has outlined a method for the removal of the capsule as a preliminary to agglutination.

An interesting modification of the resistance of bacteria to agglutination and other antibacterial antibodies is seen when bacteria are cultivated in immune sera. If grown in broth containing a sufficient concentration of the serum a culture may not only become highly resistant to agglutination but may fail completely to absorb agglutinins. The agglutinability and capacity to absorb agglutinins eventually return after cultivation on ordinary media.

Dead bacteria agglutinate well, although somewhat more slowly, if killing is done by the addition of 0.25 per cent. carbolic acid or preferably 0.1 per cent. formalin. If killed by heat the temperature should not exceed 55° C. Heating to 60° C. diminishes their agglutinability.

Dreyer found that if a twenty-four-hour bouillon culture of B. coli required 1 part of agglutinin to agglutinate it, then if heated to 60° C. it required 2.3 parts; if to 80° C., 18 parts; if to 100° C., 24.6 parts. He found the surprising fact that long heating of the culture restored to some extent its ability to be agglutinated by smaller amounts of agglutinins. Heated thirteen hours to 100° C. the culture was agglutinated by four parts.

**The Development of Agglutinins.**—Appreciable agglutinins do not develop until toward the end of the first week of infection, and the agglutinin content tends to rise if infection continues, reaching its acme at the height of the disease or somewhat later. Animals show the development of agglutinins in about the same length of time after immunization is begun. Each successive injection tends to stimulate the production of further agglutinins until a considerable concentration is present.

Different varieties of microörganisms vary in their agglutinogenic capacity. Thus the typhoid-paratyphoid group have a high agglutinogenic value, the streptococcus-pneumococcus group a relatively low power. Infection by members of the former causes a relatively high agglutinin content, whereas infections by the latter cause the production of only a relatively low agglutinin content. The same result is obtained after intensive immunization of animals, an antityphoid serum giving agglutination in dilutions of thousands or tens of thousands being easily obtained, whereas an antipneumococcus serum agglutinating above 500 to 600 is only exceptionally produced.

The amount of infection or injected antigen bears a relative proportion to the amount of agglutinins produced. As the amount of antigen is increased there is an increase of agglutinin production, but the latter increase gradually diminishes until even a very marked increase of antigen gives little associated increase in agglutinin.

**Practical Applications of the Agglutination Reaction.**—The agglutination reaction controlled by agglutinin absorption is the method employed for the determination of the identity of culturally similar microörganisms. By this means we have determined, for instance, that all races of B. typhosus are alike; that is, that B. typhosus is a distinct type or entity. In this connection it is worthy of note that a group of bacteria may differ culturally, for instance, in their fermentative reactions and still be found to be identical agglutinatively. The agglutinative method likewise allows us to separate culturally similar members of a definite bacterial species into subgroups, an example being the pneumococcus, of which we have three distinct types, and also a large group of agglutinatively dissimilar varieties.

In some instances, as already noted, we must, because of the marked degree of cross or group agglutination shown by related groups, resort to absorption before differentiation into groups is possible.

It is obvious that having knowledge of the kind above outlined we can then utilize the agglutination reaction to identify a definite type such as B. typhosus or determine into which subgroup a bacterium belongs, for example, the pneumococcus. With certain groups we shall know that the subgroup cannot be determined with certainty unless we employ the absorption method (meningococcus). With other species we will be satisfied with morphological or a cultural identification because we do not possess the necessary knowledge for further agglutinative identification or because the knowledge we have indicates the existence of a multiplicity of races. Finally, we may not be able to employ agglutination for identification because the bacterium (at least most strains) does not agglutinate well or because of its character (clumping and adhesiveness, etc.) a good suspension is difficult or impossible to make (B. anthracis). In utilizing the direct method for identification the precautions are: (1) The serum employed must be of high titer to overcome the tendency to resist agglutination shown by freshly isolated strains. The serum from infected human beings is usually unsatisfactory for this purpose because the titer is much lower than that obtainable by intensive immunization of animals. (2) The range of specific and group (including normal) agglutinins must be known, that is, determined by tests using homologous and related types. (3) The unknown type must agglutinate considerably beyond the range of group action before identification is considered sufficient for practical purposes.

The reverse application, viz., the determination of the kind of agglutinins present in a patient's serum, can be utilized for the diagnosis of disease. In this method we utilize a known bacterium to identify the agglutinins. It should be clearly understood that this method by itself does not possess final or absolute value. Thus we test a person's serum and find a considerable content of "typhoid" agglutinins. Unless we have isolated an infecting typhoid bacillus we cannot prove that they are not group agglutinins, developed because of an infection by B. paratyphosus or some other similar but infrequent type. Taking the findings, however, in connection with clinical facts and our knowledge that the majority of continued fevers of typhoidal character are (in this vicinity) due to B. typhosus the presence of agglutinins becomes the strongest possible evidence of the existence of an infection due to B. typhosus. If we suspect the infection to be due to a paratyphoid type we can determine whether B. typhosus or B. paratyphosus "A" or "B" is agglutinated in higher dilutions. The presumption is that the type agglutinated in highest dilutions is the infecting type. This is only a presumption, however, as paradoxical results may occur, as we have said, due to a high group agglutinin content or oversensitiveness of one culture. Finally, it is possible that a fourth related type may have been the infecting organism. This is remote, because cultural work has shown that these three types cause practically all the "enteric" fevers. Evidently we are interpreting our agglutination results in the light of our bacteriological and clinical knowledge of the disease. In the last-mentioned instances we can arrive at a greater certainty by applying the absorption technic. Assume that B. typhosus is the only type that will absorb the agglutinins for all three bacilli. This gives us an almost complete certainty when interpreted on the basis of the same knowledge utilized in the previous deductions. It does not give us complete certainty, as the absorption may have been of group agglutinins stimulated by a fourth variety. To reiterate, *the absorption method is only completely conclusive when we have the stimulating homologous type of bacterium for control.*

The direct agglutination reaction for diagnostic purposes has only a limited application. It can only be applied in diseases in which agglutinins are freely produced. Even here its practical value may be lessened because other simple methods are applicable earlier in the disease. Before it can be applied to a specific disease we must first determine the range of agglutinins normally possessed by uninfected individuals; knowing this we designate a minimum diagnostic dilution for a positive test. Although this method may be of little value in the early diagnosis of such acute diseases as cholera, it may be of the greatest value in making a *post facto* diagnosis. The agglutination test is chiefly used in typhoid and paratyphoid fevers. The use of the typhoid-paratyphoid vaccine creates a difficulty.

**Technic.**—The agglutination reaction may be observed in the hanging drop, using the microscope ("microscopic method"), or observed in the test-tube with the eye alone or aided by a low power lens ("macroscopic tube method"), or on a glass slide ("slide agglutination method"), a modified macroscopic method.

**Obtaining the Serum or Blood.**—Small amounts of serum may be obtained by pricking the finger or ear and collecting the blood in a Wright capsule. If the serum is not clear after clotting, the clot and red cells can be thrown down in the centrifuge. One cubic centimeter of blood can be obtained in this way. (See Chapter XII.)

Larger amounts of blood may be obtained from a vein. Collection by exsanguination is the method commonly employed with animals. The blood is collected in a tube or bottle, slanted until firmly clotted and the tube or bottle stood upright for the serum to separate. If desired every bit of serum of the centrifuge is used. A small amount of dried whole blood is collected by allowing the blood to drop on stiff paper or preferably on a glass slide. Two drops should be collected.

**Dilutions.**—With very small amounts of serum the capillary tube method of Wright may be employed (see p. 220). For large amounts the ordinary laboratory pipettes can be employed. The range and number of dilutions will depend on the specific purpose. The following suggestions may be of help to the beginner. If from the original serum we prepare a 1 to 10 dilution (one part of serum plus nine parts of physiological salt solution) and then prepare from this a 1 to 100 dilution, from this a 1 to 1000 dilution and from this a 1 to 10,000 dilution, etc., the intermediate dilutions are easily prepared thus:

| | | | | | |
|---|---|---|---|---|---|
| 1 to 10 dilution . . . | 1 part | 1 part | 1 part | 1 part | 1 part |
| plus saline . . . . | 1 part | 2 parts | 3 parts | 4 parts | 5 parts, etc. |
| gives a dilution of . . | 1 to 20 | 1 to 30 | 1 to 40 | 1 to 50 | 1 to 60 |

The same scheme holds for the other decimal multiples. Intermediate dilutions are similarly prepared, thus, 1 part of 1 to 10,000 dilution plus one-tenth part of saline gives a 1 to 11,000 dilution, etc.

In setting up the reaction we usually add the bacteria in suspension, and (see below) this makes a further dilution. The dilution thus obtained is the final dilution for recording purposes.

Dried blood is diluted by adding water to the drop. This dissolves out the hemoglobin-tinged serum and the dilution is estimated from the color. Each examiner should educate his eye by making a few accurately measured dilutions and note the color. Thus, a 1 to 10 dilution is a distinct cherry red color. Subsequent dilutions are made by mixing a loop of this with the appropriate number of loops of saline to give the higher dilutions of serum required.. The addition of an equal loop of broth culture to the serum dilutions doubles the dilution. The dried blood drop should not be broken up too much or the fine debris will be carried into the final hanging drop and injure the preparation..

The dry blood method has the great advantage of convenience and is the method employed by nearly all laboratories for the Widal reaction for the diagnosis of typhoid. Although the dilution by color is relatively inaccurate because of differences in hemaglobulin content it is sufficiently accurate for practical purposes. As already stated, one not familiar with the color shades should make known dilutions of blood for comparison.

**Technic of Microscopic Reaction (Widal Reaction).**—Preliminary dilutions of the blood or serum are prepared one-half the strength of the final dilutions desired. A loop of each dilution is placed on separate cover-slips, as well as a loop of the diluent. Then at the side of each drop is placed a loop of the culture. The two drops on each slip are then mixed with the platinum wire. This results in a doubling of each of the blood dilutions. Hollow-ground slides, the edges of the concavities having been smeared with vaselin, are warmed and placed over the cover-slips so as to seal the drop in the concavity. Warming the slides softens the vaselin so that is spreads better. The seal must be complete or the drops will dry up. The slide is then turned face up and the reaction observed under the No. 6 objective (magnification about 500 diameters). The drops must hang freely, that is, they should not be too large so that there is contact with the hollow of the slide. The drop containing diluent and culture alone is the "control." This must be carefully observed to eliminate spontaneous agglutination and as an index of the motility when a motile bacterium is employed.

A broth culture incubated for eighteen hours is usually employed in the microscopic reaction. When employed for the diagnosis of disease a "standard" "culture" is employed, that is, one of known agglutinability, otherwise the results will not be comparable. Such a culture should be one that, due to cultivation on artificial culture media, has reached a constant level of ease of agglutinability.

The beginner is apt to use too heavy a culture. The broth culture may give more delicate and sharper results when diluted by adding sterile broth.

The slides may be kept at room temperature, 20° C. or at incubator 35° C. The reaction proceeds more rapidly at the higher temperature.

If the reaction is observed through the microscope in a hanging drop a formation of clumps is seen which, if it takes place rapidly, reveals the reaction almost completed at the first glance, that is, most of the bacilli are in loose clumps and nearly or altogether motionless (Fig. 79). Between the clumps are clear spaces containing few or no isolated bacilli. If the reaction is a little less complete a few bacilli may be found moving slowly between the clumps in an aimless way, while others attached to the clumps by one end are apparently trying to pull away, much as a fly caught in fly-paper struggles for freedom. If the agglutinating substances are sufficiently present, but still less abundant, the reaction may be watched through the whole course of its development. Immediately after mixing the blood and the culture together it will be noticed that the bacilli move more slowly than before the addition of serum. Some soon cease all progressive movement, and it will be seen that they are gathering together in small groups of three or more, the individual bacilli being still somewhat separated. Gradually they close up the spaces between them and clumps are formed. According to the completeness of the reaction, either all of the bacilli may finally become clumped and immobilized or only a small portion of them, the rest remaining freely motile, and those clumped may appear to be struggling for freedom. With blood containing a large amount of agglutinating substances all the gradations in the intensity of the reaction may be observed from those shown in a marked and immediate reaction to those appearing in a late and indefinite one by simply varying the proportion of blood added to the culture fluid.

**Pseudoreactions.**—If too concentrated a solution of dry blood from a healthy person is employed a picture is often obtained which may be mistaken for a reaction. Dissolved blood always shows a varying amount of detritus, partly in the form of fibrinous clumps and prolonged microscopic examination of the mixture of dissolved blood with a culture fluid shows that the bacilli, inhibited by substances in the blood, often become more or less entangled in these clumps, and in the course of one-half to one hour very few isolated motile bacteria are seen. The fibrinous clumps alone, especially if examined with a poor light by a beginner, may be easily mistaken for clumps of bacilli. Again, the bacilli may become fixed after remaining for one-half to two hours by slight drying of the drop or the effect of substances on the cover-glass.

In order to help the student to thoroughly understand what comprises a reaction, Wilson prepared a set of drawings which are here reproduced. (Figs. 80–85.)

In making the hanging drop to be examined it is necessary to have it of such a depth that it will show at least three focal planes, otherwise the examination will be incomplete and unsatisfactory.

Fig. 79 shows a microscopic field of the top of a hanging drop of a normal bouillon culture of typhoid bacilli. The organisms are freely motile. This represents the control drop used for comparison with the drop of the same culture to which has been added a little of the blood of a person suspected to have typhoid. Note these points in Fig. 80; the organisms are evenly distributed throughout the field, except at the edge of the drop, where they are gathered in great numbers; they show great activity here, seemingly trying to crowd to the very edge. This attraction is probably due to the action exerted on the organisms by the oxygen in the air, which naturally exerts positive chemotaxis on all aërobic organisms.

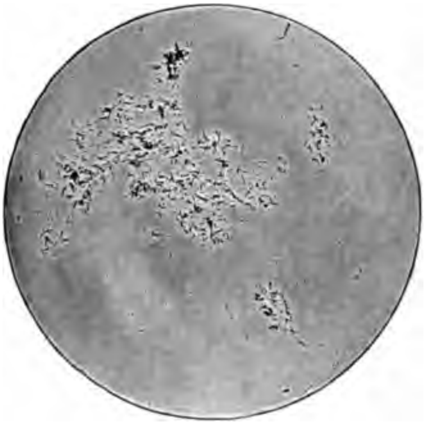

Fig. 79.—Widal reaction. Bacilli gathered into one large and two small clumps, the few isolated bacteria being motionless or almost so.

Fig. 81 shows a cross-section of the drop represented in Fig. 80, and it will be noticed that the bacilli are evenly distributed throughout the drop, except at one place in the focal plane *a* and again in the focal plane *c*.

It sometimes happens that there is a substance adhering to a supposedly clean cover-glass which attracts the bacilli to that point, where they appear as fairly well-defined clumps, more or less like the true clumps due to the agglutinating substance in typhoid blood. The increase in organisms at the bottom of the drop in the focal plane *c* is easily accounted for by the fact that gravity naturally carries the dead and non-motile organisms to the bottom, these frequently assuming the character of clumps.

If a field can be found in any focal plane of the hanging drop free from clumps one can be quite sure that any clumping present is not due to any agglutinating substance which necessarily will affect organisms in every focal plane.

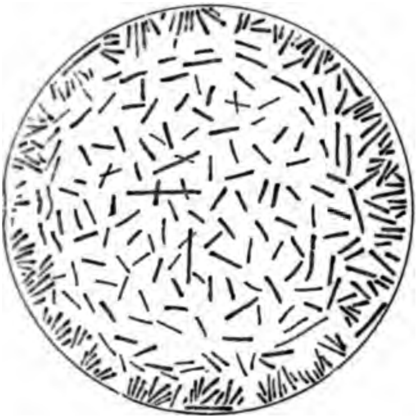

FIG. 80.—Microscopic field, showing the top of a hanging drop in a normal typhoid culture.

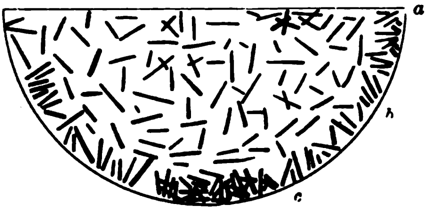

FIG. 81.—Microscopic field, showing a cross-section of the drop in Fig. 80.

Fig. 82 shows the microscopic appearance of the top of a drop in which the reaction is present. Notice first that the organisms have been drawn together in groups and that the individuals of each group appear to be loosely held together. Viewed under the microscope these clumps are practically quiescent, there being very little movement either of the

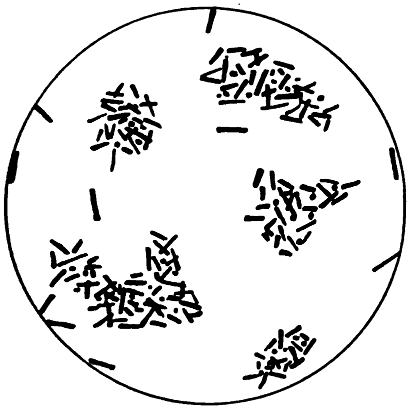

FIG. 82.—Microscopic field, showing the top of a drop with the typhoid reaction.

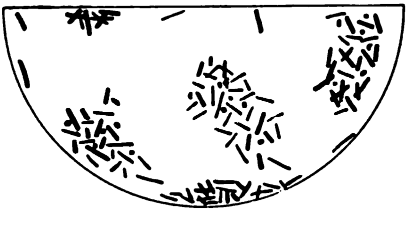

FIG. 83.—Microscopic field, showing a cross-section with the drop in Fig. 82.

individual organisms or of the clump as a whole. The edge of the drop is practically free from organisms, showing that the air no longer exerts any influence on them.

Fig. 83 shows a cross-section of the hanging drop shown in Fig. 82. The clumps are evenly distributed throughout the drop, with perhaps some increase in the numbers and compactness of the clumps at the bottom.

Fig. 84 shows the microscopic appearance of the top of a hanging drop of a bouillon culture to which has been added some blood of a patient suffering from a febrile condition not caused by typhoid infection but which exerts a slight non-specific influence on the typhoid organisms. It will be seen that the reaction is incomplete and that there are many organisms at the edge of the drop. The air exerts the same influence on the bacilli that it did before the addition.

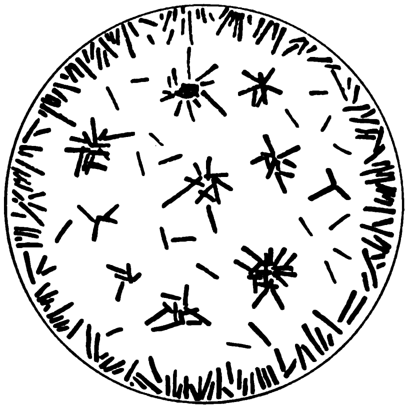

Fig. 84.—Microscopic field, showing the top of a drop of culture with reaction not due to typhoid.

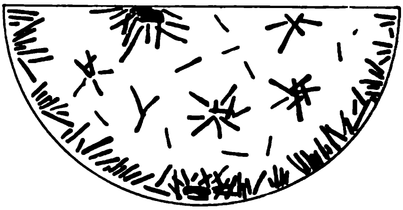

Fig. 85.—Microscopic field, showing a cross-section of Fig. 84.

Very frequently these clumps have the appearance of being built up around a piece of detritus present in the clump. All the organisms comprising the clump seem to have retained part, at least, of their motility, those on the edges being particularly motile so far as their free ends are concerned.

When motility is very much inhibited these clumps have a peculiar trembling movement, which is like the molecular movement described by Brown.

Fig. 85 shows a cross-section of the drop represented in Fig. 84. Note the same character of the clumps in every focal plant: the large number of motile bacilli and the number attracted to the edge of the drop by the air.

**Technic of the Microscopic Tube Method.**—The suspension employed may be a broth culture or a suspension in saline of the growth from an agar slant. In the case of bacteria which autolyze easily (meningococci) the suspensions should be heated to 56° C. to destroy the autolytic ferment. Such suspensions may be preserved for future use by adding a preservative, such as 0.25 per cent. carbolic acid. The suspension should not be too dense. A very slight clouding gives the sharpest results. Broth cultures to which 0.1 per cent. of formalin has been added make very satisfactory antigens. (See below.) Prepare the initial serum dilutions 10 times more concentrated than will be the desired end dilutions. Place 0.1 c.c. of each dilution in a small chemically clean (p. 89) test-tube and 0.1 c.c. of saline in another for a control. To each of the tubes add 0.9 c.c. of the bacterial suspension, shake the tubes and incubate in the water-bath or incubator at 37° to 55° C.

If agglutination takes place it is shown first by a change in the appearance of the suspension. Instead of the uniform opacity there develops a ground-glass appearance. If one uses a lens one can see very fine clumps. As the reaction proceeds the clumps become more easily visible. If after incubation for two or three hours the tubes are allowed to stand, preferably in the ice-chest overnight the clumps fall to the bottom of the tube. If the reaction is complete the supernatant fluid will be clear. In settling, the agglutinated bacteria form a broad film over the bottom of the test-tube, whereas the sedimentation of non-agglutinated bacteria results in a compact button-like sediment.

A satisfactory way to check the character of the sediment is to revolve the tubes between the palms of the hand. The swirl of the clumps leaves no doubt as to the character of the sediment. Occasionally with non-motile bacilli, either because the serum is of low titer or because the bacilli agglutinate badly, the fluid may be clear, but slight or no coherent clumps will form. Such results may be due to specific agglutination but they are difficult to read with any degree of certainty.

The control tube should always be carefully inspected to exclude spontaneous agglutination.

**Comparison of Tube and Microscopic Slide Methods.**—The reaction is the same in both and one is as reliable as the other. The ice-box readings (macroscopic) are apt to be higher than those obtained by microscopic examination. For diagnostic examinations in which haste is necessary and small amounts of serum are available, as in typhoid fever, the microscopic method is preferred. When a delay of twenty-four hours is no handicap and the serum is abundant as in tests for glanders in horses the macroscopic tube-test is generally used. Dead cultures are more frequently used in the macroscopic method because the motility here is of no importance.

**Dreyer Method of Macroscopic Agglutination.**—The basis of this method is the employment of a standardized antigen. This makes more uniform and comparable results possible. The method was suggested primarily as a means by which the rise or fall of agglutinins could be accurately determined. Thus, Dreyer believes typhoid or paratyphoid fever can be differentially diagnosed in the vaccinated because the rise or fall of the infection agglutinins is marked within short periods, whereas the agglutinins due to vaccination will be relatively stable during a similar period. To be able to determine a rise or fall with accuracy it is obvious that a standardized antigen must be employed.

The antigen is prepared by subculturing daily in broth for about ten days. Then flasks of broth are inoculated and incubated for twenty-four hours. Add 0.1 per cent. of formalin (40 per cent. formaldehyde solution), place on ice for several days, shaking repeatedly. The culture is then standardized for opacity and agglutinability. Dilutions of the culture are compared with dilutions of a standardized culture and the degree of greater density of the new culture determined by taking the mean of at least six successive selections of tubes of similar density. The culture is diluted accordingly with physiological salt solution containing 0.1 per cent. formalin and tested for agglutinability. This is done by comparing the results of agglutination with that obtained with a previously standardized culture. The new suspension is then given a factor of agglutinability based on comparison of the highest dilutions showing agglutination to the naked eye. The dilution of the standardized culture is to that of the new culture as the factor of the former is to that of the latter. The original standard had a factor of 1, and to make results with subsequent antigens comparable they must be corrected according to this factor.

If one possesses no standardized culture the optimum opacity may be determined by setting up agglutinations with various dilutions of the antigen and determining which gives the sharpest readings. One can always standardize back to this original antigen and can at any time transfer the results in terms of another standard culture.

Dreyer recommends incubation at 55° C. for two hours. For further details, including the methods Dreyer uses in expressing the results, reference is made to the article by Davison.[1] We have found the use of formalinized broth cultures of standardized opacity of the greatest value in carrying out extensive experimental work with members of the typhoid, paratyphoid and dysentery group. The use of agglutination antigens thus standardized is preferable in determining the curve of agglutinin response to vaccines in man or in animals, especially when the agglutinin response is used as a presumptive indication of the antigenic value of the vaccine. Exceedingly sharp readings are possible if the tubes are placed on ice overnight. The sharp results are due apparently to the sensitiveness of the antigen as well as its dilution, which results in fewer grades of intermediate reactions.

**The Macroscopic Slide Method.**—This method allows a rapid diagnosis of colonies from plates inoculated with suspected material, such as feces, throat cultures, etc., and can be employed in examinations for cholera, typhoid, paratyphoid and dysentery bacilli as well as for meningococci. A highly potent serum, whose specific and group agglutinating strength is known, should only be used or false positive results will be obtained; hence the method is not of much worth in clinical diagnosis of blood.

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[1] Jour. Am. Med. Assn., 1916, lxvi, 1297; also Fennel: Jour. Am. Med. Assn., 1918, lxx, 590.

The method is as follows: A loopful of saline solution (as control) and one of the highly potent specific serum in low dilution are placed on one slide and a sufficient amount of the suspected colony to give a slight turbidity is added to each. Flocculation begins in the serum almost at once if the organism tested is specific. A negative reaction is not exclusive, as relatively inagglutinable strains may be encountered, although with highly potent serum there is nearly always some evidence of a reaction. Colonies apparently typical but not distinctly agglutinable should be fished for further identification. Colonies giving a positive reaction should also be fished for verification, unless experience has shown that the serum used does not give false positive results with allied types.

**Agglutinin Absorption Technic** (see p. 190).—The basic validity of an absorption experiment depends on the standardization or selection of an appropriate absorbing dose of antigen and the demonstration of the completeness of absorption by testing the absorbed serum in relatively very low dilutions. To determine the proper dose one should test the serum first with its homologous strain and determine approximately the dose of bacteria necessary to remove all the agglutinins. In standardizing the dose one may utilize a suspension of recorded density or utilize a certain volume of packed bacteria as measured in the tip of a graduated centrifuge tube. In the former case the serum is diluted to 1 to 5 to 1 to 15; in the latter case the serum is added to the packed bacteria and salt solution added to give the dilution desired. The degree of initial dilution will depend on the titer of the serum. There is an error in calculation in both instances, as the dilution is actually lower than recorded because of the volume of bacteria. This error, however, is in the direction of making a positive result more conclusive. After mixing, the tubes are placed in the water-bath for two or three hours. They should be shaken every ten minutes to keep the agglutinating bacteria in contact with the serum. The mixtures are then usually placed on ice overnight to complete the absorption. The tubes are then centrifuged until the supernatant fluid is clear. This is then utilized for the further dilutions, the lowest dilution for the test of absorption should be equivalent to at least 10 per cent. of the original specific titer or still better 5 per cent. In every absorption test the homologous strain must be included as a control. A slight amount of deterioration of the serum occurs during the process of absorption, the degree of which will be controlled by tests with a non-absorbing type. Both low and higher dilutions of the absorbed serum must be employed to eliminate error due to prezone action.

The most stringent adherence to the above suggestions is necessary if error is to be avoided. Thus, if we have two closely allied types A and B, we find on absorption that B will tend to reduce the specific titer of A serum for A. If the absorbing dose of B is too large and the subsequent test dilutions too high an apparent specific absorption of A agglutinins will result. If, however, even with an excessive absorbing dose the dilutions were brought down to 5 per cent. or less of the original titer, agglutination of A but not of B would be observed. This reduction of specific agglutinins can be limited very much by careful attention to the absorbing dose. There is considerable latitude between the smallest absorbing dose of the homologous and the dose of a heterologous type causing marked reduction of the specific titer. At least two to three times the minimum absorbing dose of the homologous bacterium may be utilized in determining agglutinin absorptive identity. If one works too closely to the minimum absorptive dose, minor differences of absorptive capacity will be noted, even with strains of such an entity as B. typhosus. Such differences have been advanced as a basis for the assumption of antigenic varieties. As a matter of fact the absorptive capacity varies less than the sensitiveness to antibody action, and is no greater than one would expect, because of individual biological differences. As we have seen, a strain may vary or be modified in relation to its agglutinative properties, and one cannot but question the stability of any grouping on minor antigenic differences. These differences are very small even when compared with absorptive differences between two very closely related but distinct varieties.

The reduction by allied types above noted is due, to some extent, to absorption of the group agglutinins. When the absorbing dose is very excessive, reduction may occur even with little related types, the bacteria acting apparently like a non-specific absorptive reagent such as kaolin.

**Agglutinins for Other than Microbal Cells.**—Hemagglutinins, that is, agglutinins for red cells, may be normally present, or specific hemagglutinins may be produced by the injection of red cells. Goat serum normally contains agglutinins for rabbit corpuscles. Iso-agglutinins for the red cells of other individuals of the same species are encountered. This is of extreme importance in the transfusion of blood from man to man.

## CHAPTER X.

# ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.

(CONTINUED.)

## PRECIPITINS.

Precipitins are antibodies which, when added to a clear protein solution, cause a precipitation. In 1897 Kraus noted that if the serum of an animal immunized against B. typhosus is added to bacterial free filtrates of broth cultures the mixture first became turbid, which change was followed by precipitation. Similar results were obtained with other antisera and their homologous bacteria.

**Nature of Precipitins.**—The properties of precipitins are very similar to those of agglutinins. They are fairly resistant, but are gradually destroyed by heating to 60° to 70° C.. Ehrlich and Bordet consider the precipitin antibody as having the same structure as the agglutinins. Ehrlich classes it as an antibody of the second order. The loss of activity of precipitins according to this conception would be due to the deterioration of the more labile zymophore group, resulting in the formation of "precipitinoids." Precipitins are specific within certain limits.

**Mechanism of the Precipitin Reaction.**—If we look upon the precipitin antigen, that is, the clear solution of bacterial proteins as simply a suspension of invisible protein molecules as contrasted with microscopically visible bacteria in an agglutination antigen, the mechanism must be similar to that of the agglutination reaction. The known facts and theories of the mechanism of one reaction apply in general to the other.

**Prezone Phenomenon.**—This phenomenon occurs with low dilutions of some precipitating sera and is directly analogous to that observed in the agglutination reaction.

**Specific (Major), Group and Normal (Minor) Precipitins.**—During immunization both specific precipitins for the homologous bacterium as well as group precipitins for closely related types are produced. The reasons for this phenomenon are the same as given under agglutination. Normal precipitins for bacterial extracts are less commonly encountered than are normal agglutinins. Horse serum is most likely to contain them.

**Development of Precipitins.**—Precipitins are not commonly demonstrable in the serum of infected individuals. This antibody is usually present in appreciable amounts only after prolonged and intensive immunization. The precipitinogen, that is, the stimulant to its production, may be live or dead bacteria or solutions of their proteins obtained by extraction or autolysis.

**Practical Applications of Precipitin Reaction.**—The precipitin reaction has only a limited application in the identification of bacteria in culture and body fluids and discharges. It is less easily applied than the agglutination reaction, because of the necessity of preparing a clear bacterial extract. Another disadvantage is that the range of precipitin action of an antiserum is relatively low. (See below, Technic.) At present the reaction is limited in practical work to the differentiation of the types of pneumococcus. An important application is the determination of the presence and variety of bacterial substance in excretions, exudates or lesions. Thus, in lobar pneumonia considerable soluble products of the pneumococcus are found in the lung, in the sputum, in the blood and thence in the urine. Advantage is taken of this fact by using the precipitin reaction to determine the type of infecting pneumococcus. Likewise, if the sputum is injected intraperitoneally into the mouse the precipitin reaction may be similarly applied to the peritoneal exudate. In the case of anthrax infections in animals, extracts of the lesions give a precipitin reaction with immune anthrax serum, thus permitting a diagnosis. (For details of methods see under these diseases.)

The precipitin reaction with the serum of the infected host, because of the absence of appreciable amounts of precipitin, has been applied practically very little in the diagnosis of disease. The serum of horses infected with glanders usually gives a precipitin reaction, and the method has been used for diagnosis. The results are less reliable than those obtained with the agglutination or other serological reactions.

**Technic of Reaction.**—A highly immune serum must be available and the range of specific and group as well as normal precipitins must be known. The antigen may be prepared from a lesion, exudate or excretion (see Pneumococcus and Anthrax) or from agar or broth cultures. If broth cultures are incubated for several days or longer the clear broth obtained by filtration or centrifuging will give a very satisfactory antigen. Similar results may be obtained by suspending and extracting the bacteria from agar cultures in salt solution or distilled water. Or the suspension may be made in decinormal NaOH or NaCl solution and boiled. This, when neutralized and cleared, gives a very satisfactory antigen. Bacteria other than the acid-fast types can be dissolved in alkaline hypochlorite solution (antiformin). Taking advantage of these facts we have devised a rapid method of preparing a concentrated antigen by adding antiformin to a concentrated suspension of bacteria, boiling, neutralizing, precipitating with alcohol, dissolving the precipitate in saline and clearing by centrifuge.[1] Pneumococci may be dissolved by the addition of bile.

The serum undiluted and diluted 1 to 5 to 1 to 25 or more is added in 0.2 c.c. amounts to small test-tubes. To this is added a similar amount of antigen. The tubes are then placed in the water-bath or incubator at 37° to 55° C. If the reaction occurs it is first noted by the appearance of turbidity. If the reaction progresses, small floccules separate which increase in size and fall to the bottom of the tube, leaving the supernatant fluid clear. The tubes may be incubated any arbitrary time suitable to the purpose in hand. With the reactions of lesser degree storage on ice overnight aids in making comparative readings. The prezone phenomena may be noted if the antigen is too concentrated.

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[1] Krumwiede and Noble: Jour. Immun., 1918, iii, 1.

The following show the range of specific and group action with two highly immune sera:

PRECIPITIN REACTIONS—PNEUMOCOCCUS SERA.

| Pneumococcus antigen. | Time, hours. | Serum types and dilutions. | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Type I. | | | Type II. | | | Type III. | | |
| | | 1-1 | 1-4 | 1-9 | 1-1 | 1-4 | 1-9 | 1-1 | 1-4 | 1-9 |
| Type I . . . | 1 | ++[1] | + | ± | − | − | − | − | − | − |
| | 2 | +++ | ++ | +\| | ± | − | − | − | − | − |
| Type II . . | 1 | − | − | − | ++ | ++ | + | − | − | − |
| | 2 | ± | − | − | +++ | +++ | ++ | − | − | − |
| Type III . . | 1 | − | − | − | − | − | − | + | − | − |
| | 2 | − | − | − | − | − | − | +\| | ± | − |

Antigen used = 0.2 c.c. Serum solution = 0.2 c.c.

[1] +++ = profuse precipitate. ++, +|, +, ±, = decreasing amounts of precipitate.

INFLUENCE OF DILUTION OF ANTIGEN ON CROSS-REACTIONS.

| Organism. | Serum dilution (anti-typhoid). | Antigen diluted 1-10 | | | Antigen diluted 1-80. | | |
|---|---|---|---|---|---|---|---|
| | | One-half hour. | One hour. | Ice-box. | One-half hour. | One hour. | Ice-box. |
| B. typhosus . . . | 1:1 | ++ | ++ | ++ | + | + | + |
| | 1:6 | ++ | ++ | ++ | + | + | + |
| | 1:12 | + | +\| | +\| | + | + | + |
| B. paratyph "A" . | 1:1 | C | ± | + | − | − | − |
| | 1:6 | − | ± | + | − | − | − |
| | 1:12 | − | ± | + | − | − | − |
| B. paratyph "B" . | 1:1 | ± | ± | + | − | ± | ± |
| | 1:6 | − | Sl. C | ± | − | − | ± |
| | 1:12 | − | − | ± | − | − | ± |
| B. sanguinarium . | 1:1 | +\| | +\| | ++ | + | + | + |
| | 1:6 | +\| | +\| | +\| | + | + | + |
| | 1:12 | ± | + | +\| | + | + | + |

Sl. C. = slightly cloudy. C = cloudy. ± = slight flocculation. + = distinct flocculation. +| = flocculation more marked. ++ = heavy precipitate. Antigen 0.2 c.c. and serum dilution 0.2 c.c. used.

The second table is given to show the influence of the concentration of antigen on the degree of cross-reaction. The precipitation ceased after dilution to 1 to 100. The last strain of fowl origin is included to show the extremes of cross-precipitation that may be encountered.

This type tends also to cross-agglutinate, very markedly with typhoid antiserum. In this instance, however, the serum agglutinated B. typhosus in a dilution of 1 to 10,000, B. sanguinarium only to one-seventh of this dilution.

The delicacy of the precipitin reaction can be increased by floating the antigen over the serum. In this case the reaction is shown by the development of a ring at the line of junction. This is feasible only when the serum is not diluted or diluted slightly and the antigen has a lesser specific gravity.

**The Nature of the Precipitin Antigen.**—As is evidenced by the methods of preparation given above, the antigen, using this term in the narrower sense of the reacting substance, resists boiling even in the presence of acid and alkalies. In fact, its resistance is so extraordinary that it is not destroyed by boiling in strong alkaline hypochlorite solution even for one-half hour or longer. Pick also found that the precipitin antigen was not destroyed by decomposition or digestion by pepsin or trypsin. Certain data indicate the existence also of a thermolabile and alcohol-soluble precipitable fraction in bacterial extracts.

Although the substance is present in greatest amounts in broth cultures after incubation of twenty-four to forty-eight hours or longer it is demonstrable in young cultures of the pneumococcus, for instance, after four to six hours. This would seem to indicate that the substance was excreted by the bacteria rather than due to autolysis alone, which in this time would have been very slight in amount.

**Other Antibodies in the Precipitate.**—Agglutinins, bacteriolysins, complement-fixing substances and protective antibodies are carried down with the precipitate. These can be dissociated to some extent from the precipitate by extraction with weak alkaline solutions. Gay and Chickering[1] have attempted to utilize these facts as the basis of a practical method for the concentration of the antibodies in antipneumococcus serum.

**Precipitins for Other than Microbal Proteins.**—Tchistovitch and Bordet showed that the injection of alien serum would result in the production of precipitins for the injected serum. Similarly, precipitins can be prepared for vegetable proteins. Further observations revealed that this reaction had species specificity, that is, the reaction occurred quantitatively highest with sera from individuals of the same species as used for injection. The degree of group reaction with sera of other species depends on the closeness of biological relationship of the species. The antiserum precipitin will react with the other albuminous secretions of the same species. By washing organs free of blood a certain degree of organ specificity seems demonstrable. One peculiar fact is worthy of note, however, the crystalline lens from fishes to man has the same protein content as determined by the precipitin reaction.

**Practical Applications.**—Although this is not within our subject a brief outline is given. Because of the marked species specificity the precipitin reaction may be employed to identify the source of blood stains for medicolegal purposes. It is also applied in the determination of the sophistication of foods.

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[1] Jour. Exp. Med., 1915, xxi, 389; also Weinstein: Jour. Immun., 1918, iii, 17.

Given a blood stain, presumably human, the first step is to prove that it is blood. If the blood cells have disintegrated, this is done by chemical means, preferably by the demonstration of hemin crystals. The extract of the stain in salt solution is used as the antigen in the test (see below). In the case of meats a salt solution extract of the meat, or, for instance, sausage, is made and used as an antigen with antibeef, antihorse, antidog, etc., sera. That giving reaction in dilutions commensurate with controls of known meat gives us our result. The antisera are obtained by several large injections of the species serum into rabbits. The technic of the reaction is based on the fact that even very dilute amounts of antigen are demonstrable. The antigen is diluted, therefore, not the serum. With very potent antisera even dilutions of 1 to 25,000 or more of the antigen will give a reaction. In the cases mentioned above an estimated initial dilution of 1 to 1000 is prepared, from which other dilutions can then be made. The antigen is then floated over the serum and incubation carried out at 37° C. The appearance of a ring at the point of contact is a positive reaction. The reagents must be clear and many controls with other antisera and known antigens are necessary to exclude error.

**Properties of Protein Antigen.**—As contrasted with bacterial antigens they are very labile. Heating or chemical treatment renders the protein less or non-precipitable. If, however, an animal is injected with moderately heated protein a serum is obtained which precipitates the heated and the unheated serum as well as split products unaffected by the native serum precipitins. The species specificity is retained. Boiling for three to five minutes, however, reduces the specificity. These facts are utilized in testing heated meats for evidences of sophistication. It is claimed that certain types of chemical alteration destroy species specificity; for instance, iodized-beef protein, when injected, stimulating a precipitin not only for iodized-beef protein but for similarly altered proteins of other species.

# CHAPTER XI.

# ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.

(CONTINUED.)

## BACTERICIDAL, BACTERIOLYTIC AND CYTOLYTIC ANTIBODIES.

In 1888 Nuttal demonstrated that normal blood had a bactericidal or bacterium-killing property without regard to the presence of phagocytic cells. He showed that serous exudates contained similar substances and found that the bactericidal action was weaker after standing and was quickly destroyed by heating to 60° C. Buchner found the same property was possessed by serum obtained after the clotting of blood and called the active substance "alexin." Pfeiffer, in 1894, showed that if guinea-pigs were first immunized against the cholera vibrio and then injected intraperitoneally with living cholera vibrios there was a rapid swelling, granulation and dissolution of the bacteria. This process could be followed by removing portions of the peritoneal exudate with capillary tubes and examining it in stained smears. A normal guinea-pig would not show this phenomenon or at most slightly, but the phenomenon could be induced even with normal pigs if "immune" serum were introduced with the vibrios. The same result occurred if the serum was first heated to 60° C. This is known as the "Pfeiffer phenomenon." The active substance, therefore, was an acquired antibody, and, as he showed, was specific, acting only on the cholera vibrio. The hypothetical substance causing this action, Pfeiffer called "bacteriolysin," or specific bactericidal substance.

Bordet then showed that the phenomenon was due to the interaction of two substances, one the specific substance, increased during immunization, which was thermostabile (50° to 60° C.). He also showed that fresh immune serum is actively bactericidal, but if this power were lost by standing or by heating it could be fully restored by the addition of fresh guinea-pig serum which possessed little bactericidal action by itself.

**The Immune Body or Amboceptor.**—The terms bactericidal or bacteriolytic are used to denote whether the bacterium is only killed or killed and disintegrated. There is no evidence that we are dealing with two distinct antibodies. The term amboceptor was adopted by Ehrlich because he believed that the antibody was a cell receptor having two haptophore groups, one with an affinity for the bacterium the other for combination with the third substance normally present in serum, which he called complement.

He termed the amboceptor an antibody of the third order. They resist heating to 60° C., but are gradually destroyed by heating to 70°

to 80° C. The amboceptor combines with the cell in the absence of complement. As with other antibodies, those produced during immunization have highly specific as well as some group action. They are also normally present to a varying degree, as noted above, in results obtained by Nuttal and others.

**The Complement.**—Complement is present in normal and in immune serum. Its quantity is not increased during immunization. It is the active substance in bacteriolysis. Its structure, according to Ehrlich, is very similar to that of a toxin possessing a haptophore group for combination with the receptor and an active or cytophile portion to which it owes its action. The latter portion is unstable, deteriorating when serum is left at room temperature for forty-eight hours or longer, and is destroyed by heating to 55° C. for one-half hour, and is very sensitive to acids and alkalis. The residual haptophore group Ehrlich terms a complementoid, and believes that it will combine with the receptor, thus preventing the action of active complement. Such evidence as is available indicates that the leukocytes are a source, if not the only source of complement. How many varieties of complement exist is an open question. Bordet believes there is only one variety, whereas Ehrlich and others have advanced what they consider evidence to prove the existence of many varieties. Complement in its action resembles ferments very closely. (See below.)

**Mechanism of Amboceptor-Complement Action.**—According to Ehrlich's hypothesis the amboceptor acts as a bridge for the action of complement. Bordet, however, believes that amboceptor combines with the bacterial cell, sensitizing it to the action of the complement. In favor of such a conception is the fact that complement shows little if any affinity for the immune body in the absence of the homologous bacterium. If the amboceptor were simply a double haptophore bridge the complement should combine with the amboceptor. Here, again, we find differences in theoretical considerations based on hypotheses of quantitative chemical combination or on the more physical theories of colloidal adsorption. The combination of the antigen with amboceptor and complement is approximately quantitative as with other antibody combinations. The most important variation in quantitative relationship is seen in the fact that if the amount of amboceptor is increased less complement is needed, whereas if the amount of complement is increased less amboceptor is needed. Even though the mixtures of bacteria, its homologous amboceptor, be incubated for prolonged periods there is no evidence of proteolytic cleavage, yet the bacteria may be dissolved. The action of the complement on the amboceptor-cell complex would seem to be upon the cell envelope and stromata, making it permeable and liberating the protein content. Amboceptor and complement have different avidities in relation to temperature; thus amboceptor will combine with the bacterial cell even at a temperature just above the freezing-point, whereas complement will only combine with the cell-amboceptor complex at higher temperatures, its optimum being at 37° C., at which temperature it also displays its highest activity. Amboceptor and complement are fixed by the substances in extracts of bacteria, even in the absence of demonstrable precipitation. (See Precipitins.)

**Determination of Bactericidal or Bacteriolytic Action.**—The activity of a serum may be determined by *in vivo* and *in vitro* methods. The Pfeiffer phenomenon in guinea-pigs is utilized for the former. The test is carried out by injecting a series of guinea-pigs intraperitoneally with a constant amount of culture and decreasing amounts of the serum. The reaction is observed by removing portions of the peritoneal exudate to determine the degree of action. The smallest amount of serum yielding a positive reaction gives us the titer of the serum.

The test-tube method is usually carried out according to the technic of Stern and Korte. To a series of test-tubes add decreasing amounts of the inactivated (heated to 55° C. half hour) serum. To each tube then add 0.1 c.c. of fresh guinea-pig serum to supply a constant amount of complement. Add saline to bring the contents of each tube to constant amount, 0.5 or 1 c.c. To each tube add a small amount of dilute suspension or broth culture of the homologous organism. Incubate for three hours at 37° C., and then make poured agar plates of the contents of each tube and incubate and determine the relative number of colonies developing. Adequate controls must be made, a salt solution control before and after incubation, an amboceptor control of the lowest dilution without complement and of complement alone. The guinea-pig serum may be bactericidal itself. The sterility of each of the reagents must also be controlled.

It is obvious that with this method, even though we did not inactivate the serum, we are titrating the amboceptor content as we are adding complement to activate the amboceptor in the high dilutions. If we wished to determine the actual bactericidal value of native serum because of its content of immune body and natural complement we should carry out the test in a similar manner employing a constant amount of fresh active serum but decreasing the dose of culture. The number of organisms in the decreasing dose may be estimated by control plating. The largest number of organisms that is killed by this constant dose of serum is the measure of the serum.

The methods are relatively difficult to carry out and unsatisfactory results are common with the plate methods because the culture or the complement employed are not satisfactory. Reduplication or comparable results are difficult to obtain on successive tests for the same reasons. The tests are not applied practically today for purposes of identification of bacteria or as a diagnostic procedure. The Pfeiffer reaction has lost its importance as a means of identification because the simpler agglutination reaction gives us the same information. Stern and Korte used the method as a diagnostic procedure in typhoid fever and found that the serum of a patient might be strongly bactericidal when the agglutination was low or doubtful. They obtained strong bactericidal action at times in dilutions of 1 to 50,000 or more. Here, again, the method is too complicated and bacteriological methods are more easily applied as a substitute for or in addition to the Widal reaction.

The methods are used primarily for experimental purposes to determine the response to injection of vaccines or to compare the bactericidal action of serums with the degree of action of other antibodies.

**Prezone Phenomenon.**—In the above *in vitro* tests it not infrequently happens that the larger amounts of serum show no bactericidal action. This might be explained as due to complementoids according to the Ehrlich hypothesis or to complement deviation or deflection as suggested by Neisser and Wechsberg. In the latter hypothesis not all the combined amboceptors would be activated, the receptors not attached to the cells blocking the combination of sufficient complement. This assumes a combination of amboceptor and antigen prior to combination with the cell, for which we have little if any evidence. The phenomenon may be due to a similar colloidal reaction, as pointed out for other antibodies. Protective substances may be present which prevents amboceptor combination or complement combination (anticomplementary action) or both, these substances becoming insufficient to cause inhibition as the serum is diluted. The fixation of complement by the precipitin combination with any soluble antigen present has also been advanced as an explanation. (See below.)

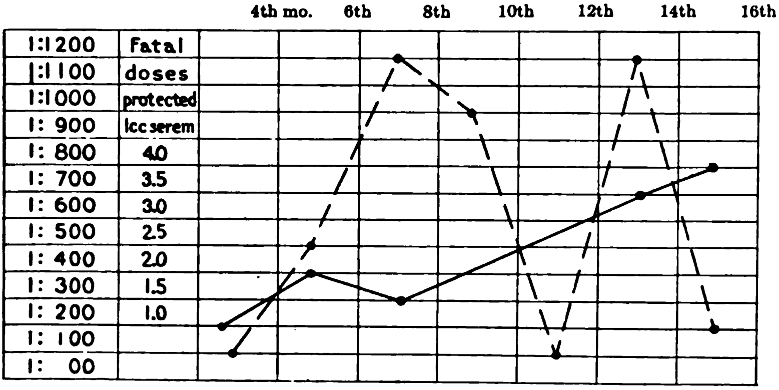


FIG. 86.—Relation of agglutinative to bactericidal power. Horse injected with culture of dysentery bacilli over a period of sixteen months.
— — — Agglutination index.
——— Bactericidal index.
• Test dates.

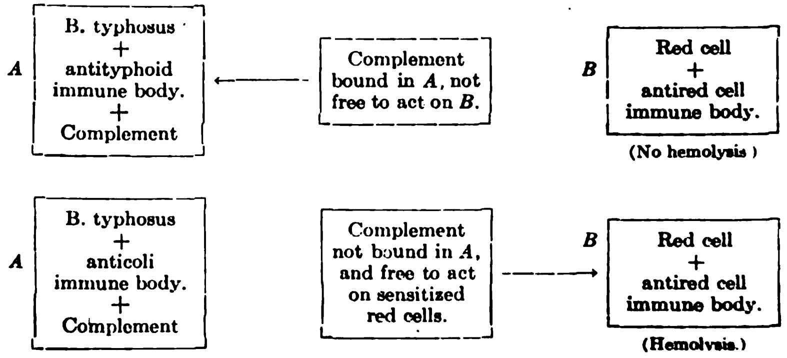
**Relation between Agglutinating and Bactericidal Power.**—In spite of proof to the contrary, the belief persists that there is regularly a parallelism between the agglutinating and the bactericidal strength of a serum. In Fig. 86 are recorded a number of comparative tests during a period of sixteen months. The experiment shows no definite relation between content of agglutinins and immune bodies. We have found in horses subjected to injections of a fixed type of pneumococcus that the agglutinating strength increases before the bactericidal strength and that it decreases while the latter is still increasing.

**Lytic Antibodies for other than Microbal Cells.**—Bordet showed that if the red cells of alien species were injected into an animal, antibodies were produced which would cause hemolysis of the cells. Although the cells were not dissolved the action of the antiserum resulted in a liberation of the hemoglobulin from the cell stromata, the latter remaining as shadow forms. It was soon evident that this action, as with bacteriolysis, was due to two substances: a hemolytic amboceptor, which resisted heating to 55° C., and whose production is stimulated by the injections and the complement, sensitive to heating to 55° C., which is a normal constituent of the blood. The amboceptor or immune body was found to be specific for the cells of the animal used for injections. Group antibodies may be present in less amounts, however, for the cells of allied species.

One species may naturally possess hemolysins for the red cells of another species. Thus normal goat serum will hemolyze rabbits' red cells. Isolysins may be normally present in the serum of one individual for other individuals of the same species. This has to be considered in selecting donors for blood transfusion. Cytolysins may be produced for cells other than red cells.

**Method of Eliciting Hemolysis and Titration of Reagents.**—As this is of importance to us primarily in a specialized procedure, "complement-fixation" (see below), it is given in the chapter on that reaction.

**Bordet-Gengou Phenomenon or Complement-fixation Reaction.**—If we take an antigen, bacterial or non-bacterial and add to it a small amount of inactivated homologous immune serum and complement (fresh guinea-pig serum) and incubate the mixture, there will result a combination of the three elements. If we have not added too much complement none should be left free. We can determine this to be so by adding red cells which have been sensitized by incubation with their homologous antiserum. There being no complement free, no hemolysis results. If we repeat the experiment and use a heterologous antigen or a heterologous antiserum, complement should not be combined. This will be shown when we add our "complement indicator," red cells sensitized with their homologous antigen, hemolysis quickly develops after appropriate incubation. These facts are diagrammatically represented as follows:


A
B. typhosus + antityphoid immune body. + Complement
Complement bound in A, not free to act on B.
B
Red cell + antired cell immune body.
(No hemolysis)
A
B. typhosus + anticoli immune body. + Complement
Complement not bound in A, and free to act on sensitized red cells.
B
Red cell + antired cell immune body.
(Hemolysis.)

As we have outlined the reaction it would appear that a positive (no hemolysis) result is wholly due to the fixation of complement by bactericidal antibodies. This is not the case. Other factors enter. We have already referred to the absorption of other antibodies by the precipitin-antigen complex. As Gay has shown, this complex will fix complement as well. Neisser and Sachs found that very minute amounts of human blood mixed with its antiserum would fix complement and suggested this method for forensic blood tests. Although the injection of blood serum may give rise to amboceptor as well as precipitins, most investigators believe the complement is bound by the precipitin-antigen complex. The complement may be bound even when no demonstrable precipitation occurs. For instance, in the presence of an excess of antigen the precipitate may not separate. The precipitin-antigen complex held in "solution" by the excess of antigen fixes complement. It is evident, therefore, that complement-fixation is a much more delicate antigen indicator, that is, the precipitin reaction.

**Application of the Bordet-Gengou Phenomenon.**—Three applications of this reaction should be apparent and theoretically possible: (1) The use of known antibodies to identify or classify unknown microörganisms; this application has been used to a considerable extent, but has many drawbacks as compared with the simpler agglutination reaction. When satisfactory agglutination antigens are not possible the reaction has considerable value. Closely allied types tend to give greater group reactions than are encountered with the agglutination reaction. These may be eliminated to some extent by careful titration of the reagents. (2) The second application is the demonstration of the presence and the identification of the antibodies in the patient's serum. This application is, therefore, a diagnostic procedure. This procedure is also employed in strengthening the evidence that a microörganism isolated from a disease is the causative agent. Naturally the presence of antibodies does not prove that it is the primary etiological agent of the disease or infection, as the antibodies might develop if the organism were a secondary infecting agent. (3) The third application is the use of the reaction to quantitatively determine the content of complement-fixing antibodies in antibacterial serums to be used for therapeutic purposes. These methods and the technical details involved are given in the chapter on Complement Fixation.

# CHAPTER XII.

# ANTIMICROBAL OR ANTIPROTEIN SUBSTANCES.

(CONTINUED.)

## OPSONINS AND PHAGOCYTOSIS.

Although it had been suggested, by earlier observers, that ingestion of bacteria by the body cells was a means by which the body destroyed bacteria, Metchnikoff was the first to experimentally prove this fact. This ingestion is followed by a digestion and is analogous to the feeding processes of unicellular organisms. Metchnikoff noticed that phagocytosis was more active in the presence of immune serum than with normal serum, but thought that this was due to the presence of leukocyte-stimulating substances in the serum. Denys and Leclef suggested that the action of immune serum might be upon the bacterium reducing their resistance of phagocytosis. By utilizing the technic of Leishman, Wright and Douglas definitely proved the existence of a substance in serum which, acting upon the bacterium, prepared it for phagocytosis. This substance Wright gave the name "opsonin."

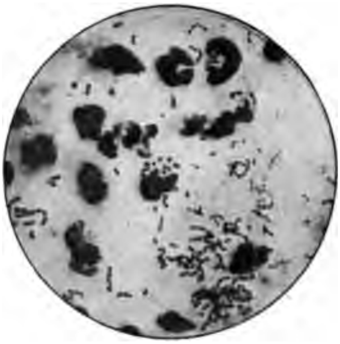


FIG. 87.—Streptococcus engulfed by leukocytes from abscess. Gentian-violet stain. × 1000. (Fränkel and Pfeiffer.)

**The Phagocytic Cells.**—Metchnikoff differentiated two varieties: the "motile or wandering" and the fixed phagocytes; the former, the leukocytes, the latter the endothelial cells, as well as certain fixed connective-tissue cells and cells of the lymphoid tissues (lymph-nodes and spleen) and of the neuroglia. The most active fixed phagocytes are the endothelial cells of the bloodvessels and serous cavities and lymph sinuses. The polymorphonuclear leukocytes he designated as "microphages," the large mononuclear leukocytes and the fixed phagocytes as "macrophages." The participation of these cells is seen in the process of inflammation. If an infection occurs, due to the streptococcus, there is an inflammatory response which is dominated by the collection of polynuclear leukocytes which are attracted ("positive chemotaxis") and pass through the wall of the capillaries to the tissues.

At the same time there is an accumulation of serous exudate. The polynuclear leukocytes as well as the local phagocytic cells attempt to dispose of the bacteria. If the infection progresses, the accumulation of cells continues and the leukocytes and tissue cells which are killed form the resulting "pus." This consists mostly of the accumulated leukocytes and serous elements.

If the infection had been due to tubercle bacilli a different type of inflammation would occur. The bacilli will be rapidly surrounded by large mononuclear cells, apparently endothelial in origin, and about the latter will develop an exudation of cells of the lymphocyte type. The polynuclear variety is not attracted ("negative chemotaxis"). Phagocytosis by the endothelioid cells and the giant cells formed from these is noticed. If the infection progresses, necrosis develops in the center of the cell mass, resulting in caseation. The polynuclear leukocytes, however, play a role in the preliminary attempt to dispose of the bacilli, as is seen in the phagocytosis when tubercle bacilli are introduced into a serous cavity.

In general infections the blood picture, total leukocyte and differential count, gives evidence of the positive or negative chemotactic character of the infecting organism. If an infection is due to an excessively virulent organism or if infection be extensive and severe a negative chemotaxis results even though the infecting agent is ordinarily of the positive variety.

**Opsonins.**—Opsonins are present in normal serum and in greater amounts in the serum of immune animals. Evidence has been advanced that the "normal" and the "immune" opsonins are not similar substances.

Thus the former are nearly destroyed by heating to 60° C. for fifteen minutes, whereas the latter seem more heat resistant, not being markedly affected by heating to 62° to 63° for forty-five minutes. The question arises, What relationship, if any, have these substances to those already studied? Careful experiment indicates that the opsonic action of normal serum is due to distinct antibodies. The action of normal opsonins is apparently due to a thermostabile (55° C.) substance present only in small amounts the activity of which is very much enhanced by other thermolabile (55° C.) substances, non-specific in character present in the serum. The latter substance is apparently the complement. The thermostabile substance is relatively specific in the sense that the absorption of normal serum by a specific organism will only remove those for itself or related organism, but not necessarily all the opsonic substances.

The immune opsonins or bacteriotropins (Neufeld) would resemble antibodies of the second order (Ehrlich) if the evidence advanced as to their heat resistance and inability to be reactivated by normal serum was conclusive. Dean, Hektoen and others, although admitting the thermostabile character of the immune opsonin, have shown that the action is enhanced by the addition of fresh normal serum, inactive in itself. It would seem, therefore, that there is no fundamental difference between the normal and immune opsonins or bacteriotropins. The apparent greater resistance of the latter may be partly because of their presence in greater concentration. In both instances the opsonic action is not completely lost on heating, thus apparently differing from the complete inactivation of bactericidal action brought about by the same means. This residuum may remain only apparently active, not being active in itself, but because the leukocytes used in the test supply traces of complement which reactivates the opsonic antibody. This hypothesis, however, rests on the still doubtful contention that the leukocytes are a source of complement. If this hypothesis were true it would explain the apparently greater heat resistance of immune opsonins, which, being in greater concentration, would require less complement to activate them. As we have pointed out, the greater the concentration of amboceptor the less complement is necessary. The fact that the opsonin value and the bactericidal action of a serum do not necessarily parallel each other quantitatively, indicates that the opsonic action is due to a distinct antibody and not due to the action of bactericidal amboceptor either alone or with complement. A final opinion, however, will be possible only when the doubtful points of opsonic action are settled.

Immune opsonins or bacteriotropins like other antibodies are specific for the stimulating microörganism. Some group action, however, may be evident, with closely related types. As with other antibodies, microorganisms exhibit a variable degree of resistance to the action of opsonins. This again is relatively proportionate to their virulence and source, and can be artificially modified. The resistance to phagocytosis may not be wholly due to resistance to the combination of opsonins. The bacterium may secrete substances which repel (see aggressins) or actually injure the leukocytes (leucocidins). As has been previously noted, capsule production is apparently a protection against antibody action. Pathogenic bacteria which have been cultivated on artificial media for some time may be spontaneously phagocyted, that is, phagocyted in the absence of serum.

**Variations in Activity of Phagocytic Cells.**—According to the facts given above, one would conclude that the increased phagocytic capacity of the cells of the immune host were wholly due to the increased opsonins. It has been shown that in lobar pneumonia the leukocytes may show an increased phagocytic power without regard to serum action. This may be due to other factors, as for instance, the age of the cell rather than to an acquired cell characteristic. Park and Biggs have shown that differences exist between the cells of apparently healthy individuals. Probably an equal degree of difference occurs with cells of the same individual at different times.

**Mechanism of Phagocytosis.**—The phenomenon of chemotaxis and of phagocytosis can be simulated with inanimate physical agents. A study of these agents indicates that chemotactic action is due to substances which lower the surface tension of the cell. This causes attraction and results in phagocytosis, thus if the leukocyte meets a substance which reduces its surface tension it flows about or engulfs the substance. After ingestion, microörganisms are subjected to the action of the endolysins or ferments by which they are destroyed and digested.

**Endolysins and Endo-enzymes.**—Leukocytes, as shown by Schattenfroh, contain bactericidal substances. These can be extracted. They differ from similar serum antibodies in that they are more thermostabile, a temperature of 75° to 80° C. being necessary to destroy them. These substances have been termed "endolysins." Zinnser showed that one could extract no greater amount of these substances from the cells of immune than from normal individuals. Endolysins are not specific. Little if any of this substance is contained in the lymphocytes and macrophages. A number of enzymes have also been obtained from phagocytic cells, those of the leukocytes being called leukoprotease. Opie obtained two proteolytic ferments in the cells of exudates, one from the polynuclear cells, active in weak alkaline solutions, the other from exudates containing large numbers of mononuclear cells, active in weak acid solutions. The leukocytes possess no lipase which, however, is present in the macrophages. The leukocyte, therefore, cannot digest acid-fast bacilli such as the tubercle bacillus, but carries them to the lymph nodes for digestion by the macrophage. In this way tubercle bacilli or other microörganisms, which resist the power of the leukocytic endolysins and endoferments may be disseminated by what is essentially a protective mechanism. Because of the presence of the above substances extracts of leukocytes may on injection have protective or therapeutic value (see below.)

**Technic of Demonstration and Measurement of Opsonic Action.**—Viable leukocytes may be obtained from sterile exudates or from the blood. In the former a 5 per cent. aleuronat suspension in a 3 per cent. starch solution in broth or a 25 per cent. solution of peptone is injected intraperitoneally into a guinea-pig or intrapleurally into a rabbit. After sixteen to twenty-four hours the animal is killed and the exudate is collected and added to about 20 c.c. of a 1 per cent. sodium citrate in an 0.8 per cent. salt solution. This is centrifuged, the leukocytes are again suspended in saline and are again sedimented, three or four times successively to wash away traces of serum. The leukocytes are then suspended in saline. Leukocytes are obtained from the blood by adding 1 part of blood to 15 to 20 parts of citrate salt solution, which prevents clotting. Small amounts, 1 c.c., may be obtained by deep puncture of the finger or the blood may be obtained from the vein by syringe. The blood of experimental animals may be used. The diluted blood is centrifuged at low speed. The red cells having the greater specific gravity are sedimented first, the leukocytes last, collecting as a creamy layer over the red cells.

The supernatant fluid is removed without disturbing this layer, and leukocytes are collected with a capillary tube. They will be mixed with red cells. The leukocytes are now placed in saline in another centrifuge tube, centrifuged and the cells washed free of serum as described above,

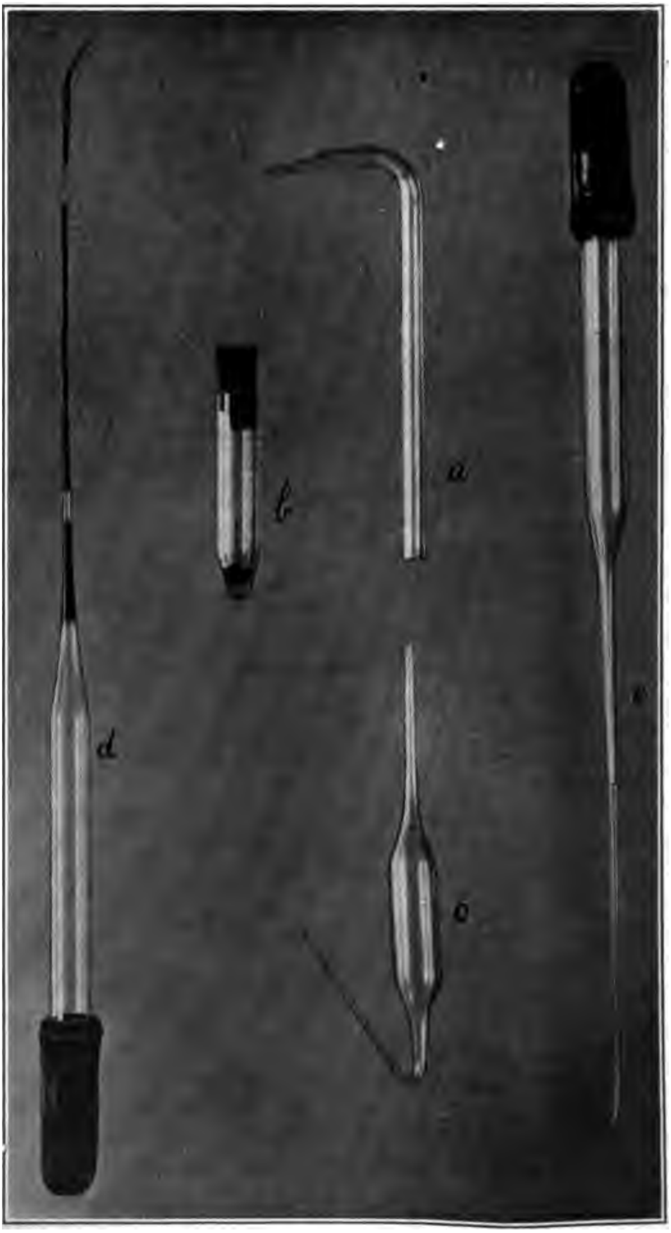


Fig. 88.—Opsonic outfit.

and finally suspended in saline. The solutions employed should be warmed to 37° C. as cold will affect the activity of the cells. Centrifuging at too high speed is to be avoided as this may clump the leukocytes. For careful work and especially if the activity of different cells is to be compared the cells in the suspensions should be counted and standardized to a definite content of polynuclear leukocytes.

The bacterial suspension may be either a saline suspension of the growth on agar or a broth culture. The culture selected must not give undue spontaneous phagocytosis nor should it be unduly resistant to opsonic action. The suspension must be a satisfactory density (see below).

The serum is collected from man by puncture, allowing the blood to flow into a Wright capsule. The blood of experimental animals is collected from the vein and placed in a test-tube to clot. If necessary the clot is loosened from the glass so that it will contract and the serum separate.

**The Method of Wright-Opsonic Index.**—This method gives a comparison between the opsonic action of a serum with that from a normal individual or the pooled serum from several normals. The latter constitutes the control or standard measure. A capillary pipette is made and marked about an inch from the end. With a rubber teat the leukocyte suspension is sucked up to this mark, then a small bubble is allowed to enter the tip, then the bacterial suspension is sucked up to the mark, a bubble allowed to enter and last the serum drawn up to the mark. We now have three equal quantities of cells, bacteria and serum, separated by bubbles. The contents are then mixed by blowing out the contents on a slide and sucking it up, repeating this several times. Bubbling must be avoided. The contents, sucked well up into the tube, the tip is sealed in the flame, and the tube incubated at 37° C. for twenty minutes or longer. The tip is then nicked with a file and the contents again mixed, a drop placed on a slide, a smear prepared, using another slide, or cigarette paper as a spreader. This is then fixed and stained and examined with the oil-immersion lens. The leukocytes are more numerous along the edges of the smears. The average number of bacteria per leukocyte is determined by counting the contents of fifty or preferably one hundred cells. This is done with the normal or control serum and with the serum for test. The result is expressed by the opsonic index thus:

$$\frac{\text{5 per leukocyte-test serum.}}{\text{10 per leukocyte-control serum.}} = \text{Opsonic index; 0.5.}$$

Before carrying out this method the bacterial suspension must be standardized so that not more than five to ten bacteria per leukocyte will be taken up in the time of incubation. This is done by preliminary tests with the suspension and dilutions of the suspension.

**Method of Neufeld.**—This method involves the determination of the highest dilution that still causes phagocytosis. Dilutions of the serum to be tested as well as of a normal serum (same species) for control are prepared. Mixtures may then be made as with the Wright technic, or 0.1 c.c. of the bacterial suspensions and serum dilutions may be mixed, incubated to sensitize the bacteria, 0.1 c.c. of the leukocyte suspension then added and the mixtures again incubated for twenty to thirty minutes. Smears are then made from each of the tubes. A salt solution control should also be included. Neufeld advises the inactivation of immune sera.

**Accuracy of Opsonic Determinations.**—In one and same smear marked differences will be noted in the degree of phagocytosis of different leukocytes. To arrive at any degree of accuracy, therefore, with the Wright method a considerable number of cells must be counted. This is clearly shown in the following table:

OPSONIC INDEX ESTIMATIONS IN FIVE BLOOD SPECIMENS.

| Cells counted. | Average number of bacteria in each leukocyte. | | | | |
|---|---|---|---|---|---|
| 50 | 1.18 | 1.88 | 1.34 | 1.42 | 1.90 |
| 100 | 1.22 | 1.78 | 1.24 | 1.42 | 1.59 |
| 150 | 1.18 | 1.62 | 1.22 | 1.44 | 1.50 |
| 200 | 1.18 | 1.51 | 1.22 | 1.46 | 1.37 |
| 600 | 1.28 | 1.62 | 1.23 | 1.36 | 1.36 |
| 1200 | 1.34 | 1.44 | 1.25 | 1.30 | 1.42 |

One difficulty with the Wright method is the selection of a normal control. Even the use of pooled serum does not exclude variation. The degree of such variation is evident from the following:

OPSONIC COUNTS IN TEST OF TWENTY-ONE NORMAL SERA WITH STOCK STAPHYLOCOCCUS CULTURE.

| | | | | | |
|---|---|---|---|---|---|
| 1 | 4.13 | 8 | 3.82 | 15 | 9.09 |
| 2 | 2.93 | 9 | 3.95 | 16 | 5.17 |
| 3 | 2.78 | 10 | 3.98 | 17 | 4.04 |
| 4 | 4.37 | 11 | 4.27 | 18 | 3.82 |
| 5 | 3.58 | 12 | 3.69 | 19 | 4.00 |
| 6 | 2.90 | 13 | 3.80 | 20 | 3.79 |
| 7 | 3.56 | 14 | 3.59 | 21 | 3.44 |

The Wright and the Neufeld methods are likely to give extremely discordant results because of actual differences among the leukocytes, the number in the suspension and the culture employed. The latter will vary according to the strain employed and differences in density of suspension will also lead to variable results. Individual observers will estimate or count slightly differently which will be a further factor. These factors become very evident when sera are submitted to four or five competent observers. Under these conditions variations of 10 to 20 per cent. are common and at times differences of 100 per cent. are encountered. In determining the opsonic content of therapeutic sera obtained from the horse, the high content of normal opsonins is a further factor of error. On the whole the method has many uncontrollable variants.

**Applications of the Opsonic Determinations.**—Wright advocated the method as a means of control of vaccine therapy. Where vaccines are injected, Wright states, there "supervenes a negative phase where there is a diminished content in protective substances. This is succeeded by a positive phase. This inflowing wave of protective substances rapidly flows out again, but leaves behind in the blood a more or less permanently increased content of protective substances. When a small dose of vaccine is given the negative phase may hardly appear, but the positive phase may be correspondingly diminished. Where an unduly large dose of vaccine is inoculated the negative phase is prolonged and much attenuated. The positive phase may in such a case make default. It will be obvious that, if we, in the case of a patient who is already the subject of a bacterial invasion, produce by the injection of an excessive dose of vaccine a prolonged and well-marked negative phase, we may, instead of benefiting the patient, bring about conditions which will enable the bacteria to run riot in his system."

"Now consideration will show that we may obtain, according as we choose our time and our dose wisely or unwisely, either a cumulative effect in the direction of a positive phase or a cumulative effect in the direction of a negative phase. We may in other cases, by the agency of two or more successive inoculations, raise the patient by successive steps to a higher level of immunity, or, as the case may be, bring down by successive steps to a lower level. We can select the appropriate time and dose with certainty only by examining the blood and measuring its content in protective substances in each case before reinoculating."

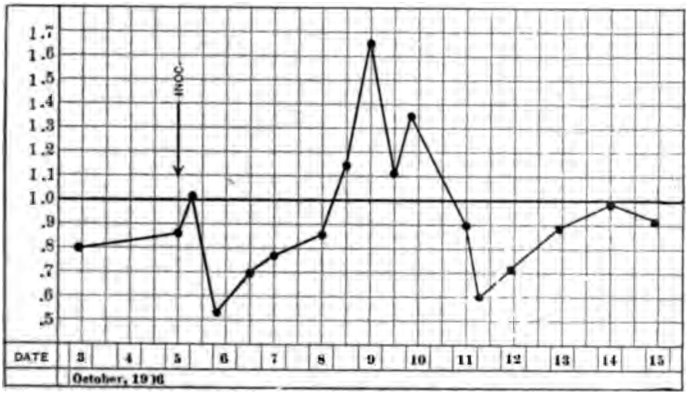


**FIG. 89.**—Opsonic curve, showing the slight immediate rise and the later negative and positive phases following inoculation. The changes here are more regular than generally occurs.

The reasons for the discontinuance of this method are several: (1) the relative inaccuracy of the method; (2) the fact that almost equal differences can be observed in normal or diseased individuals from day to day without regard to injections of vaccines (Fig. 90); (3) injections were based, not on the index at the time of injection, but of the previous day because of the exigencies of the opsonic method; (4) the development of a negative phase is apparent, not real; (5) equally satisfactory results were obtained, controlling dosage by the degree of focal and systemic reaction.

The determination of the opsonic content of immune horse serum to be used for therapeutic purposes has been very generally employed especially for the standardization of antimeningococcus serum. The method is far from satisfactory for the reasons given and is no longer employed as a routine test. The determination of the presence of an increased opsonic content has been utilized to a limited extent as a diagnostic procedure. On the whole the method is only applicable to special investigative work and the results must be scrupulously controlled and the results verified by many repetitions of the test.

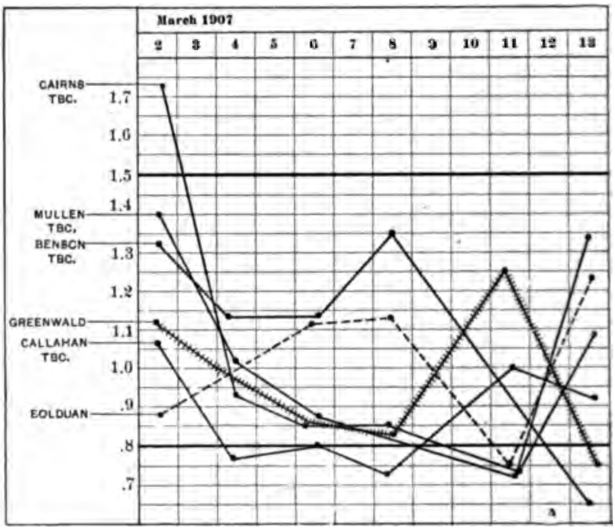


FIG. 90.—Dotted and crossed lines, normal persons. Continuous lines, tuberculosis.

**Opsonins for Other than Microbal Cells.**—As with other antibodies, these may be easily produced. Those for red cells hemopsonins have been mostly employed for experimental purposes in determining the factors of opsonic action.

**Leukocytic Extracts.**—Hiss and Zinnser prepared a leukocytic extract for therapeutic purposes. They injected rabbits intrapleurally with an aleuronat suspension, killed them after twenty-four hours, added the exudate to citrate solution and finally extracted the cellular sediment with distilled water.

Experimentally they showed that such extracts possessed protective value in infected rabbits. In many cases they, and others, obtained favorable therapeutic results in man in the treatment of pneumonia, staphylococcus infections, meningitis, influenza and other types of infection. Zinnser believes the use should be confined to local infections. The extract is given in 10 c.c. doses every four to six hours. Although beneficial results are apparent in some cases, other cases have shown no benefit. On the whole the results have not been striking. Large amounts of extract may be prepared by using horses.

**Use of Antibacterial Serums for Therapeutic Purposes.**—Sera having a high antibody content also show considerable protective value on animal injection. Likewise, therapeutic value is indicated for such sera in that they prevent or delay the death of experimental animals even when the serum is administered after the infecting dose of bacteria is given. The relative effect, however, is directly proportionate to the shortness of time which elapses between infecting and serum dose. This is partly due to limitations of such animal experimentation. These are imposed by the fact that we are not reproducing the natural disease in the animals but causing an infection, the only reliable criterion of the severity of which is the death or recovery of the animal. Because of these facts antibacterial sera have been utilized in man for both prophylactic and therapeutic purposes. (See Part III.)

**General Methods Used in Production of Antibacterial Sera.**—*Immunization of Horses.*—Subcutaneous injections have been practised in the past, but this method has certain objections which have led to the almost general adoption of intravenous inoculation. With the latter method, a high degree of immunity is more quickly established, the amount of culture required less, and certain complications avoided, such as the development of abscesses after meningococcus vaccines. The frequency of inoculation will depend on conditions. Daily intravenous injections alternating with periods of rest is the most rapid method of producing antisera, giving 3 to 5 successive daily injections followed by several days to a week's rest. We have found injections once a week adequate in maintaining the titre of the sera after the maximum is reached.

**Preparation of Vaccine.**—This varies with the bacterium employed. The easily growing types are cultivated on agar and suspended in saline. Meningococci are grown on glucose agar. Streptococci or pneumococci may be grown on agar or in broth. If the latter, the cocci thrown down and then washed with saline solution and finally suspended in saline for injection. Injection of the broth or culture ingredients is to be avoided, as serious reactions or even sudden death may occur when large amounts are given. This has been attributed to the peptone-like substances. The relation of the virulence of the injected microorganism to antibody production is not well understood. It is usually advised, for instance, that the pneumococci be kept fully virulent for mice. We do not believe this to be necessary. Stock strains are used for the other species of microörganisms.

**Selection of Strains.**—This will depend on whether a univalent, polyvalent or a serum against representative strains is desired. In the case of meningococci the strains should cover all agglutinative variants. With pneumococci, a serum against both Types I and II can be produced with antibody content against each as active as in a univalent serum, but as a rule the horse does not stand such injections for any considerable period. With streptococci, the representative hemolytic strains are employed as we have little reason to include the viridans type as they are practically heterogeneous and even sera from autogenous strains have given no results in chronic endocarditis. Torrey's ten strains of gonococci are usually employed. For dysentery sera the representative types are employed.

**Horses.**—Healthy horses are selected. (Deformities or crippling is no bar.) They are tested for glanders with mallein and by the complement-fixation reaction. An injection of tetanus antitoxin is given every few months.

**Dosage.**—The dosage depends on the toxicity of the strains employed and the temperature and general reaction of the horse as well as his general health.

Specific dosage, therefore, cannot be given. Depending on the type of bacterium $\frac{1}{20}$ to $\frac{1}{10}$ of an agar slant or 1 to 2 c.c. of a broth culture is the initial intravenous dose. This is then gradually increased until a maximum is reached. When injections are given only once a week, larger doses may be given than when given daily. After the injections, temperatures should be taken frequently. The optimum dose causes a sharp febrile reaction to about 104° F., but the temperature returns to normal in eight to twelve hours. A chill may occur. The maximum dose is the amount that will maintain the required antibody content and the balance of antibodies if several strains are employed, but if this requires doses which give excessive reactions, the horses' period of productivity will be short. Desensitization, "so-called" as advised by Dopter and Amoss and Wollstein, that is, the preliminary inoculation of a small dose, has been of no value in our hands. With most bacteria live cultures may be used from the start. Streptococci and pneumococci had better be killed at first. Autolysates (salt-solution suspensions covered with toluol and stored on ice) of meningococci were advised at first by Flexner. Amoss and Wollstein found that the autolysate alone gives rise to serum of poor quality. This is contrary to our experience. We maintained the therapeutic value of the serum from a horse for a period of over six months with the injection of autolysate alone. More observations are needed, its advantage is its convenience, its disadvantage the relatively greater reactions it produces in the horses so that the dose should be smaller than its equivalent in live cocci.

**Titration of Sera.**—*Standards.*—The methods employed and the standards adopted, vary to a great extent, as the problem involved is much more complex than with toxin-antitoxin methods. The methods possible of application are dilution titration of the antibodies, agglutinins, opsonins and bactericidal content and the determination of the protective value when injected with cultures. The agglutinin content is chiefly of value with typhoid and dysentery, although the content in these antibodies may decline after prolonged inoculation without, so far as we know, a coincident fall of other antibodies. The agglutination reaction is of some value in determining the probable antibody balance where several or many types of strains are employed as with dysentery and meningococci, this being the guide as to the pro-rata amounts to be injected into the horses. The value of this method is naturally influenced by the tendency of the horse to produce common agglutinins. Opsonic determination is only of comparative value. It cannot be used as a basis for a standard as it is open to serious error and variations beyond control. The bactericidal titration has found only limited application. The complement-fixation reaction has been of value in titrating meningococcus serum; and a rough standard that not more than 0.002 c.c. shall be required to give complete fixation, using a mixed antigen, is employed in several laboratories. The protective value of meningococcus serum, using white mice (Hitchens and Robinson[1]), promises to be of value. For pneumococcus serum protective experiments with white mice offers the best method According to Cole, 0.2 c.c. of serum should protect against 0.1 c.c. (Type I) or 0.01 c.c. (Type II) of a broth culture (of which 0.000001 c.c. kills mice) when culture and serum are injected simultaneously. Protection experiments can also be applied to streptococcus serum, although difficulties may be encountered in raising the virulence of the strains.

On the whole the methods are far from satisfactory, titration of one antibody does not necessarily give us any information as to the content in other antibodies. Where many representative types are employed, the balance not only as regards one antibody, but possible variations in comparative content of different antibodies against the individual strains still further complicates matters, as well as insufficient knowledge of the actual immunological relationship of these strains. Protection tests are satisfactory where the bacterium is truly septicemic for the test animal, less so where invasion is less marked (meningococci) and of least value where death is due essentially to endotoxins. Protection tests, furthermore, only give us information concerning the strain used, and in the case of meningococci, for instance, we know little as to the degree of cross-protection with strains having agglutinative relationship. Furthermore, there is only partial knowledge as to the relation of the total antibody content to protection and finally to therapeutic effect in man.

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[1] Jour. Immun., 1916, i, 345 and 355.

**Bleeding.**—Bleeding for therapeutic sera are made with trocar and rubber tube into 2-liter Erlenmeyer flasks having a large flat wire egg-beater to support the clot. After the flask is about half-full, it is tilted on the side where the wire is inserted. The flasks may be stood up after the clot is firm. The serum separating after twenty-four and forty-eight hours is drawn off with siphon. All operations must be aseptic.

**Serum.**—The collected serum is placed in a sterile vessel and trikresol added, drop by drop, with vigorous stirring to prevent precipitation, until 0.4 per cent. is added. In two to three weeks, after which further fibrin separation usually ceases, it is passed through a Berkefeld filter to remove any contamination occurring at the time the trikresol was added. The trikresol will prevent any multiplication of the contaminants prior to filtration. Instead of trikresol one may add several cubic centimeters of chloroform per liter and shake, the excess settles out with any separated fibrin and is avoided when the serum is drawn up in the blotting apparatus. The sera must be kept in the refrigerator at all times and tested to determine its sterility before being issued.

**Concentration.**—This is not practised as it is with the antitoxins. To test the different fractions and determine which to use is, to a great extent, impracticable until we have better methods for standardization and a clearer knowledge as to what antibodies are essentially of therapeutic value.

**Technic of Protection Test.**—The culture used should be highly virulent or at least as virulent as possible, for the test animal (mouse, guinea-pig or rabbit). If not of high virulence, one should attempt to raise its virulence by animal passage. The minimal lethal dose of the culture is then determined using either the intravenous or intraperitoneal route. The actual test is then carried out by mixing an arbitrary volume of serum with increasing amounts of culture or an arbitrary amount of culture with decreasing amounts of serum. The mixtures are injected intraperitoneally (mice or guinea-pigs) or intravenously (rabbits) and the animals kept under observation. The following tables will serve as examples:

PNEUMOCOCCUS PROTECTION TEST. CONSTANT AMOUNT OF SERUM.

| Mouse. | Serum. | Broth culture. | Result. |
|---|---|---|---|
| 1 | 0.2 c.c. | 0.05 c.c. | Survived. |
| 2 | 0.2 c.c. | 0.10 c.c. | " |
| 3 | 0.2 c.c. | 0.20 c.c. | " |
| 4 | 0.2 c.c. | 0.30 c.c. | Dead 48 hours. |
| Control A | 0 | 0.0000001 | Survived. |
| Control B | 0 | 0.000001 | Dead 24 hours. |

PNEUMOCOCCUS PROTECTION TEST. CONSTANT AMOUNT OF CULTURE.

| Mouse. | Serum. | Broth culture. | Result. |
|---|---|---|---|
| 1 | 2.0 c.c. | 0.2 c.c. | Survived. |
| 2 | 1.0 c.c. | 0.2 c.c. | " |
| 3 | 0.5 c.c. | 0.2 c.c. | " |
| 4 | 0.25 c.c. | 0.2 c.c. | Died 48 hours. |
| Control A | 0 | 0.0000001 | Survived. |
| Control B | 0 | 0.000001 | Died 24 hours. |

If agar cultures are employed (meningococcus) the fractions of the surface growth are the measure employed, or better, the number in suspension may be determined by actual count.

*Every animal that dies must be cultured to exclude spontaneous infection* due to its normal septicemic types. If the test organism is invasive its presence can be demonstrated by smear or culture from the heart's blood. Further identification will be necessary when the injected type and the normal septicemia type are similar.

We have stated that the protection test gives its best results when the bacterium is highly virulent.

It is doubtful even with extremely virulent bacteria that multiple proportions hold between serum and culture when the mass of culture reaches a certain level.

The average of some experiments of Dochez on pneumococcus protection show this, thus 0.1 c.c. of serum would protect against 0.5 c.c. of culture, 0.25 c.c. against 0.8 c.c. but 3 c.c. was necessary to protect against 1 c.c. This would seem to be due to endotoxin action not neutralizable by the serum. With moderately virulent cultures the limits may be still narrower, so that experimental errors in direct as well as in cross-protection tests to determine the relationships of cultures one to the other are easily made.

**Cross-protection and Strain Identity.**—The error of assuming strain identity on the basis of insufficiently controlled protection tests is frequently heard. Thus, if antiserum A will protect against Strains A, B, C, etc., this is advanced as proof of identity. As a matter of fact it is only an indication of group relationships. The following schematic presentation of the results of Avery will show this very clearly:

PNEUMOCOCCUS PROTECTION TESTS.

| | Variety of culture. | | |
|---|---|---|---|
| Variety of anti-serum. | Type II. | Subgroup IIa. | Subgroup IIb. |
| Type II . . . . . . | Protects | Protects | Protects |
| Subgroup IIa . . . . | No protection | Protects | No protection |
| Subgroup IIb . . . . | No protection | No protection | Protects |

In other words the reverse phenomenon must be observed before conclusions are drawn. The question arises: would even mutual protection be complete evidence of identity? If this were encountered the relative degree of cross-protection which naturally should always be carefully controlled might reveal differences. Theoretically at least, there is no reason to believe that apparently complete mutual cross-protection might not occur with two closely related but not identical strains. If this is theoretically possible it again brings us to our previous theorem that *antibody absorption is our ultimate criterion.* This has been discussed primarily under agglutination, because agglutinin absorption is usually the expedient method since we can control the technic more easily and because quantitative estimations can be more easily and accurately made. The same principles, however, are applicable to the absorption of other antibodies.

**Other Methods of Determining Protection.**—Animals may be immunized with a vaccine and the protective power conferred measured by the resistance of the animal to a fatal dose (or multiples) of a virulent organism of the same type. This method may also be employed to study the degree of cross-protection, conferred by related types. Here again cross-protection is not necessarily a criterion of identity.

# CHAPTER XIII.

# HYPERSENSITIVENESS.

## ENZYMES AND ANTI-ENZYMES, AGGRESSINS.[1]

In preceding chapters we have considered the production of immunity through the introduction of antigenic substances. We have now to consider a phenomenon which seems at first to be the antithesis of immunity. Thus, if a very small amount of bacterial or other protein be injected into a guinea-pig, a change occurs so that while the first dose produces no noticeable reaction a second injection of the same protein given ten to fourteen days later will cause symptoms of shock or even death of the animal. This phenomenon Richet called "anaphylaxis." This reaction in the guinea-pig has been studied in detail with the hope that the evidence acquired would help explain somewhat similar phenomena encountered in infectious diseases as well as the reactions following the introduction of repeated injections of antitoxins or sera, ingestion of certain food or drugs (idiosyncrasies), etc. Furthermore, any reasonable hypothesis advanced in explanation of these phenomena would seem to apply to the development of the common symptoms (fever, etc.) of infections.

The terminology as well as the classification of these altered reactions to foreign proteins is far from satisfactory on account of the many contradictory hypotheses advanced in explanation. It would seem wiser to limit the term anaphylaxis to the acute symptoms developing after an injection of protein, because of the sensitization induced by the previous injection or injections of the same protein, a phenomenon seen best in the experimentally induced condition in animals. One reason for this is that there is increasing evidence that this is a separate phenomenon. Another is that the use of the term for all the reactions seen in man following the administration of antitoxins and serums had led to undue fear of untoward reactions, as in the minds of most the term implies acute shock.

**Anaphylaxis in Guinea-pigs.**—Theobald Smith found that when he attempted to give an injection of horse serum to a guinea-pig which had been used for antitoxin testing some weeks before, that striking symptoms developed and that fully half of the animals died in thirty minutes. Untreated animals showed no reaction from the same dose.

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[1] For fuller discussion and bibliography see:

A. F. Coca: Hypersensitiveness, Tice's Practice of Medicine.

V. C. Vaughan and W. J. Vaughan: Protein Split Products in Relation to Immunity and Disease.

Also references cited previously, Chapter VII.

Rosenau and Anderson and Otto studied this phenomenon and showed conclusively that the antitoxic content of the serum was not a factor, but that the symptoms were wholly due to the serum proteins. It was found that an incubation period was necessary before the pig became hypersensitive and that as this period became longer the larger the initial or sensitizing dose. Very small amounts of serum sufficed to sensitize, even as little as 0.000001 c.c. Larger doses (approximately 1000 times the minimum sensitizing dose) are necessary to elicit symptoms. The size of dose depends on the rapidity of absorption. Thus a small dose will kill when given intravenously; a larger dose is needed if injected intraperitoneally and still more if giving subcutaneously. The rapidity of development and the extent of symptoms developing after the second injection depend on the sensitiveness of the animal and the size and mode of administration of the dose. The guinea-pig first shows restlessness, then scratches at the mouth, coughs and develops spasmodic or rapid breathing. Urine and feces are discharged. The guinea-pig then falls on its side, breathing becomes difficult or arrested and spasmodic or convulsive movements supervene. The convulsions are usually followed by the death of the animal. Associated with these symptoms there are a fall in temperature, a leukopenia, a diminution of complement in the blood, and a delay or loss of blood coagulability. On autopsy the lungs are markedly distended, due to stimulation and contraction of the smooth muscle of the small alveoli, thus retaining the inspired air. This explains the respiratory symptoms noted before death. The heart continues to beat for some time after death. The contraction of smooth muscle is due to peripheral irritation.

**In Rabbits.**—It has been estimated that rabbits are $\frac{1}{100}$th as sensitive as guinea-pigs. Symptoms of anaphylaxis may be produced easily, but acute anaphylactic death occurs only irregularly. Several injections of serum are needed to sensitize the animal. Shock is most constantly produced when 1 or 2 c.c. are injected at five-day intervals and then after three days giving daily intraperitoneal or intravenous injections of 0.2 c.c. for two weeks or more. The shock injection 2 c.c. is given five days later by the intravenous route. The symptoms elicited are less marked than with the guinea-pig and the intense dyspnea is absent. Death is due to cardiac and vascular disturbance. The pulmonary arterial system is so contracted that perfusion of salt solution even under high pressure is impossible.

Besides acute anaphylaxis, Arthus has demonstrated a peculiar local effect following repeated doses of serum. If several or more injections of serum are given a rabbit, subsequent subcutaneous injections will frequently cause severe local reactions which may result in local necrosis (Arthus's phenomenon). This is not a cumulative effect, as previous subcutaneous injections caused no reaction even when given in the same place, whereas the later reaction occurs wherever there is an area of injection.

**In Dogs.**—Dogs may be sensitized with two injections of serum (first subcutaneous, second intravenous) five days apart. A shock dose of 20 to 30 c.c. given intravenously several weeks later will cause symptoms and death. Vomiting or retching and evacuation of the bowels are among the earliest symptoms. The circulatory disturbance is apparently due to engorgement of the liver resulting in a great accumulation of blood in this organ and in the portal vessels. This would account for the anemia and low pressure in the general circulation.

**Dependence of Symptoms on Contraction of Smooth Muscle.**—Schultz has demonstrated that the hypersensitiveness of smooth muscle is the main factor in anaphylactic response. In the guinea-pig this is shown by the contraction of the bronchial musculature. In rabbits and dogs this bronchial phenomenon is not marked, possibly because the bronchi are relatively poorly supplied with muscle, but the circulation disturbances in the dog and the rabbit are explainable on the same basis. The cause of the disturbance is the same, though there is a difference in the site of action. This effect on smooth muscle can be demonstrated on excised muscular organs such as the uterus.

**Sensitization by Enteral Introduction.**—In the preceding paragraphs sensitization by the parenteral administration has only been mentioned. Sensitization induced by feeding, has been reported by Rosenau and Anderson. Other observers have had contradictory results. It would seem that success depended on whether unchanged protein reached the large intestine, from which it can be absorbed.

**Passive Anaphylaxis.**—In contrast to the active sensitiveness, hypersensitiveness of one animal may be transferred to another by the injection of the blood or serum of the first animal. This is not only possible with animals of the same species (homologous, viz., guinea-pig to guinea-pig), but with animals of different species (heterologous, viz., rabbit to guinea-pig). Hypersensitiveness is transferred to the offspring of sensitized mothers; thus, young guinea-pigs may be sensitive for five or six weeks or longer after birth. Hypersensitiveness to specific toxin cannot be passively transferred. There is no specific hypersensitiveness of the ordinary kind to exotoxins.

Unless the anaphylactogen (see below) and the immune serum are injected together anaphylactic symptoms cannot be induced in a guinea-pig by the injection of antigen, until some time (four to six hours) after the immune serum has been given (see below.)

In the rabbit and dog it may be induced immediately after injection of the immune serum.

**The Anaphylactogen.**—This term is applied to the substance which induces the state of anaphylaxis. All evidence indicates that only proteins which can act as antigens may become anaphylactogens. The proteins may be of animal or plant origin, including microörganisms and their extracts. Such proteins must be alien to the animal injected or at least alien to his circulation.

Probably the whole protein molecule is necessary to induce hypersensitiveness. Proteins split by digestion or chemical agents lose the sensitizing property. Heating diminishes or destroys the sensitizing action of proteins in proportion to the degree of coagulation caused by the heating. The ability to produce anaphylactic shock may be lost before the ability to sensitize, because minute residues of the original protein would still suffice for sensitization. Vaughan believes that the protein possesses a non-specific toxic portion and a specific sensitizing portion. He obtained two such fractions by treatment with an alcoholic solution of alkali. The sensitization by the one fraction could equally be used as an argument that all of the protein molecules had not been cleaved. The anaphylactogen, at least with native proteins and bacteria, is specific, that is, only the same protein which was used for sensitization will cause anaphylactic symptoms. This specificity follows, as a rule, the biological origin, as has been noted with other antigens.

**Similar Non-specific Phenomena.**—The products of protein cleavage when injected intravenously in normal animals are toxic. An example has been noted, viz., that obtained by Vaughan. Peptone likewise causes the symptoms of anaphylaxis. Bordet showed that the addition of agar jelly to guinea-pig serum and subsequent incubation at 37° C. rendered the serum toxic. In fact, many substances, chloroform, colloidal silica, kaolin and even distilled water, will render normal or immune serums toxic. This toxicity manifests itself by causing anaphylactic symptoms. The same effect may be noted after intravenous injection of such substances, the toxification of the blood occurring *in vivo*. Novy has found that blood in the precoagulation stage is likewise toxic. These results have an extremely important bearing on explanatory theories of anaphylaxis. Our acceptance of a theory will depend on whether or not we agree that the similar physiological response is proof that the poison in these non-specific phenomena is the same as in specific anaphylaxis.

**Anti-anaphylaxis or Desensitization.**—If animals survive the second dose of protein they are for some days relatively very insensitive to the protein. The injection into the guinea-pig of one or more small doses of the specific protein during the period of incubation has the same effect. This condition of desensitization is only temporary, sensitization again developing after several weeks. The serum of an anti-anaphylactic animal does not confer this property to a second animal; in fact the opposite occurs, the second animal becoming passively sensitized. The injection of an immune serum (specific for the protein) prior to the dose of protein will interfere with development of anaphylactic symptoms. A relative resistance to anaphylaxis may also be induced by the injection of non-specific substances, proteins, peptones, etc., as well as with drugs, atropin, chloral, etc.

**Theories of Anaphylaxis.**—Two theories have been advanced which assume that the toxic agent is a digestion product. Vaughan believes that the first injection sensitizes the animal by stimulating the production of specific zymogen which when activated will cleave the protein on second injection, the products of this cleavage being toxic. The zymogen according to this conception would be an amboceptor-like antibody which would be activated by the complement. Friedberger maintains the same views based on his observations that the toxin, inducing anaphylactic shock, develops in test-tube mixtures of antigen and its antiserum; the term anaphylotoxin is used to designate the shock-inducing poison.

This conception then involves the idea that amboceptor and complement activity results in proteolytic cleavage. Jobling found no evidence that amboceptor and complement will cause proteolytic cleavage. Nor is there reason to believe that there is produced some other specific antisubstance having actual proteoclastic activity. To sustain his theory, Vaughan concludes that the anaphylotoxin and the poisons resulting from proteolytic cleavage are the same, because they cause the same physiological effect. By the same reasoning he would be forced to assume that the toxic products in a mixture of serum and a non-specific non-protein substance (see above) where no protein antigen is available for cleavage is again the same poison. Friedberger's idea that complement is essential to the reaction is also untenable for similar reasons.

Jobling in his earlier work met these objections by the hypothesis—that the digestion products come from the serum and not from the antigen. This digestion resulting from the depression or neutralization of the antitryptic substances which would allow the serum-protease to digest the serum. Non-specific substances as mentioned above would cause this effect because of their neutralizing action. Specificity could be assumed on this basis to be due to the fact that the antigen-antibody complex would possess this neutralizing action.

Both digestion theories fail in our opinion to explain the quick response especially of excised smooth muscle. Dale employing the reaction of the uterine muscle, has questioned whether toxic digestion products could be produced within the time between contact of antigen and muscle response. The rate of reaction is equal to that of a preformed protein poison such as histamin. Digestion theories fail to interpret the delay in passive sensitization of guinea-pigs.

Novy and De Kruif have elaborated a theory, drawing an analogy between the production of anaphylotoxin and the mechanism of blood coagulation. This latter results from the change of fibrinogen into fibrin through the so-called fibrin ferment. They assume the existence of a more or less labile matrix in the blood which is changed by a catalyzer into anaphylotoxin. The two phenomena are similar, therefore, in that a labile substance undergoes rearrangement resulting in tautomeric modifications, on the one hand the insoluble fibrin, on the other, the soluble anaphylotoxin. This theory assumes that the non-specific substances noted above give rise to the same poison as is active in specific anaphylaxis. The only difference is that the antigen-antibody complex is the catalytic agent in specific anaphylaxis. This theory also fails to explain all the phenomena, especially the delay in passive sensitization. It does seem to eliminate the doubtful aspects of the digestion theories.

A moderate toxicity of the blood has been demonstrated by Novy for animals sensitized and shocked by relatively large doses of antigen or by non-specific agents. The question arises would this be so if the minimal shock-inducing dose had been used. Weil transfusing normal dogs with even the whole volume of dogs dying or dead of anaphylactic shock uniformly failed to demonstrate any increased toxicity. If the toxic products were formed in the circulating blood, one would expect to be able to demonstrate at least some toxicity.

If the digestion theories or that of Novy assumed the cell as the site of toxin production, the time factor raised by Dale would still be a difficulty.

All the theories thus far given do not take into consideration the fact that the organs of an immune animal are hypersensitive although the animal as a whole is not. This as well as the fact that the injection of immune serum specific for the protein to be used will prevent anaphylactic shock, indicates strongly that an excess of antibodies in the circulating blood acts as a protective barrier, preventing the antigen from reaching the cells.

Because of the facts given above, the opinion is gradually being adopted, that the site of the production of the toxic substance is in the cells. This is spoken of as the cellular theory. Many observers believe that the antigen combine with the cellular antibodies (sessile) and that the combination in some way injures the susceptible cells. Such a theory would be compatible with the delay in passive sensitization, the latent period being assumed necessary for cellular combination by the introduced antibody.

The question as to the antibody involved is not easily answered. The production of anaphylaxis in guinea-pigs in which complement is suppressed has been advanced as proof that complement is not involved in the reaction. The absence of demonstrable complement in the blood is not necessarily a demonstration of its absence in the cells. Most of the adherents of the cellular theory believe that the precipitins are involved in the reaction. This, at least, is not susceptible to direct proof. As we have seen, these antibodies are not easily produced in demonstrable amounts, and they are certainly not demonstrable in guinea-pigs sensitized with minute quantities of antigen. There is evidence, however, indicating that the capacity of serum to confer passive hypersensitiveness is proportionate to its precipitin content.

The fact that the washed specific precipitates resulting from antigen-antiserum mixtures will passively sensitize guinea-pigs does not necessarily indicate that the precipitin is involved. The precipitate as we have seen, will carry down other antibodies as well. If a cellular theory as outlined is accepted, the specific anaphylotoxin must be assumed to be distinct from the toxin produced in the test-tube by admixtures of specific antigen and antiserum by serum and non-specific substances, as well as from the products of protein digestion. A theory that the first injection causes a physical change in the cells, rendering them hypersensitive to a second injection has been advanced.

On the whole one cannot but conclude that no hypothesis thus far advanced is satisfactory. However, the value of these hypotheses as stimulants to experimental observation should not be underrated.

**Anaphylaxis and the Symptoms of Infection.**—Vaughan's theory of anaphylaxis has received such wide attention and acceptance because it seemed to explain in a satisfactory manner the toxic symptoms of infection caused by bacteria producing no demonstrable extracellular poisons. According to this conception the digestion products of the microbal protein would be the active agent. Carrying this conception further, the poison would be non-specific in character, being developed in the cleavage of any protein. Vaughan has shown that fever and the accompanying symptoms noted in all infections can be reproduced by the injection of protein cleavage products, the character of the fever depending on the size and number of doses injected. The hypothesis of Friedberger is very similar. The modified theories of anaphylotoxin production of Jobling or that of Novy could be similarly adapted as an explanation. Although there is strong doubt that digestive products play any role in the development of the anaphylactic reaction, the objections raised would not hold true in relation to the symptoms of disease. In the absence of more opposing evidence it seems probable that digestion products, although not due directly to antibody action may play a partial role at least in the production of disease symptoms. In this connection, however, it must be remembered that the mere autolysis in water of some microörganisms, for instance, the pneumococcus, will yield an acutely toxic substance. The question arises again, however, are such substances directly or indirectly poisonous. If, as seems implicated in such hypotheses, antibodies are necessary for the development of the poison it is difficult to understand the symptoms associated with very acute or fulminating infections.

**Toxin Hypersensitiveness.**—Because of certain phenomena, some investigators believe that there develops at times a hypersensitiveness to soluble or exotoxins, comparable to some extent with the hypersensitiveness to bacterial protein. As has been noted in the previous chapter, if an M. L. D. dose of toxin is divided into ten or twenty parts and one part injected every day or every other day, death will take place when about 40 to 60 per cent. of the M. L. D. dose is given. It has also been observed that if several small doses of toxin are given, fifteen to twenty days later, additional doses of as little as $\frac{1}{100}$th M. L. D. will cause death. Suggested explanations are that either certain cells stimulated by the previous injections have developed a greater avidity for the toxin or that some cells naturally possess a greater avidity. In either case, there being little toxin, they absorb practically all of the toxin or as much as when a larger dose is given at one time.

Behring has made the statement that horses under immunization possessing large amounts of antitoxin in their blood, showed hypersensitiveness to the toxin. These animals, however, did not develop symptoms of tetanus or the specific change due to diphtheria toxin, depending on which was being used in immunization. Doerr, Pick and others have pointed out that as we never inject toxin in a pure state, the symptoms were most probably due to the associated meat extractives peptone and products of bacterial autolysis present in the toxic broth.

This is our opinion. It seems to be sustained by the appearance of some type of reaction when horses are immunized by the injection of broth cultures of other non-toxin-producing bacteria. We have immunized over 700 horses and have never encountered a reaction suggestive of toxin hypersensitive after antitoxin had developed. We have found that only those animals possessing no natural antitoxin will show such a phenomenon but they will no longer react in this way when antitoxin appears in the blood. This is true also for small animals.

We have already referred to the long delay which intervenes between the injection of toxin and the development of antitoxin in the guinea-pig or rabbit. This seems to have misled many observers, thus Loewis and Meyer reported that small animals which have no natural antitoxin will show no response to a single injection of a toxin-antitoxin mixture, but will respond with antitoxin development if a second dose is given some weeks later. Our own observations show that all animals will in time develop at least some antitoxin from a single dose. As has been pointed out, in the previous chapter, if antitoxin is normally present or if it has developed, a subsequent injection of toxin or of a toxin-antitoxin mixture will cause a quicker and greater response than in cases where no antitoxin is present. Hypersensitiveness of a different kind exists, therefore, in the sense that the ability of antitoxin production is increased.

**Hypersensitiveness in Man.**—Phenomena of hypersensitiveness in man may be observed after both the enteral and parenteral introduction of many and varied substances. These inducing substances may be divided into two groups, antigenic and non-antigenic, viz., those which stimulate and those that do not stimulate the production of demonstrable antibodies. The degree of response to such substances is largely personal idiosyncrasy and the symptoms, however diverse the agent, have a great deal of similarity. Coca has suggested the term allergy, introduced by Von Pirquet, be limited to describe these individual peculiarities. A special term would certainly be advisable if it is eventually proved, as much of the evidence now indicates, that even where the inducing substance has antigenic properties, the reaction is not due to antigen-antibody combination or at least that the antigenic property has no bearing on the reaction.

**Serum Reaction.**—The untoward symptoms elicited in man by the introduction of serums with or without antitoxins may be divided into those following the initial injection and those following the second or later injections. These reactions have nothing to do with the antibody content of the injected serum.

Following the first injection three types of reactions may be noted: (*a*) collapse, with or without fatal outcome, (*b*) a symptom-complex called "serum sickness," (*c*) local necrosis. Each of these forms of response may follow the second or later injections.

**Collapse or Death.**—This rare accident has been noted nearly always after the first injection. The symptoms develop quickly after administration. In about 1 to 20,000 primary injections of antitoxin, alarming symptoms develop; in about 1 to 50,000 injections death results. The symptoms are those of extreme dyspnea and collapse. The dose may be small, in one instance about 1 c.c. (500 units) of antitoxin being given subcutaneously. Kerley reports a case of known hypersensitiveness where the dose was gradually increased until 4 minims were given, this resulted in alarming shock. The individuals showing this type of reaction are commonly those subject to "hay fever, or asthma," developing attacks especially in the neighborhood of horses. Nearly all of the children dying after serum shock are cases of "status lymphaticus."

A chill more or less severe is observed in about 40 per cent. of the cases after the intravenous injection of low-potency antitoxin, even when injections are given very slowly and the material warmed to the body temperature. In the Willard Parker Hospital the routine administration of antitoxin by the intravenous method has revealed that this probably depends on some special form of the protein, possibly upon a fine flocculent precipitate. With the best product less than 1 per cent. of injections produce a chill.

In some instances the intravenous administration of antitoxin or serum, several weeks or longer after an initial injection, which caused no marked reaction, has resulted in alarming symptoms of collapse. This effect almost never follows a second subcutaneous injection. In other instances, frequently repeated intravenous injection of serum has developed instead of a desensitization a hypersensitiveness so marked that even small amounts of serum would give a sharp reaction. Such conditions are relatively very infrequent.

**Serum Sickness.**—The incidence of this type of reaction varies widely in different series of cases, from 10 to 60 per cent. or more. The size of dose will influence the percentage incidence. Because of the lower protein content and also because of the heating, the concentrated globulin preparations of antitoxin cause a relatively low incidence. Following the first injection of serum or antitoxin there is an incubation period varying from three hours to twenty-four days. More commonly the period ranges from three to twelve days. The symptoms are primarily a skin eruption, edema, slight albuminuria, variable both in incidence and in degree, enlargement of the lymph nodes and pain and tenderness, and pain in the joints. The eruption is very variable in character. A local eruption appears earlier than the general eruption. On the second or later injections, the period of incubation may be absent or shortened "immediate or accelerated reaction," although this does not always occur. This condition is not serious, and in the many instances gives no greater discomfort than any itching rash. Some samples of serum or antitoxin uniformly cause a skin eruption (scarlatinaform) earlier than others. The longer incubation periods are more frequently followed by urticarial rashes. It would seem from this that there were different reaction-inducing substances in serum or antitoxin.

Von Pirquet and Schick who have studied this condition in detail, believe that the reaction is due to antigen-antibody combination. They point out that the average incubation period coincides with the time of first appearance of precipitins in experimental animals. Likewise, they believe that the immediate or accelerated reactions following later injections are explained by the presence of developed or developing antibodies. This explanation, however, does not include the fairly common occurrence of a short incubation period. There is no parallelism between the presence of demonstrable precipitins in human beings and the appearance or severity of the reaction; in fact, precipitins may be present without allergy.

**Local Reactions.**—In very rare instances the primary injection of antitoxin leads to local necrosis. Although this occurs with extreme infrequency it should be a warning against injection under the breasts. When repeated injections are given, a final subcutaneous injection somewhat more frequently results in a sharp local reaction which may go on to necrosis. This may occur not only with serum, but also with rabies vaccine. The resemblance of this form of reaction to the Arthus phenomenon is marked. The necrosis is not due to bacterial contamination but the necrotic area may become infected and serious or fatal results ensue.

**Desensitization to Serum.**—Instances are noted with some frequency where first injections have caused reactions and following injections given several days or weeks later have produced little or none. This would seem to indicate a desensitization. As already noted, different batches of bactericidal or antitoxic serum will vary widely in their rash and temperature-producing qualities, and this may have been a factor in the development or non-development of symptoms. The administration of small doses of serum, prior to a first injection or prior to subsequent injections of those known to be sensitive, has not the regularity of desensitization noted in experimental animals. The observation of Kerley already noted is an example of such failure. Divided doses may fail to give a reaction or repeated small doses may apparently induce a tolerance, but this is no proof that we are inducing the mechanism of desensitization so uniformly producible in experimental animals.

**Prevention of Serum Reactions.**—Such individuals as give a strongly suspicious history may be tested cutaneously for evidences of hypersensitiveness. The appearance of a wheal indicates that the person will show a fairly immediate serum reaction, such as rise of temperature or urticarial rash, but is no indication of the probable severity. The absence of a skin reaction indicates, but does not prove that there will be no reaction. The safest procedure, however, is to inject the serum or antitoxin in divided doses every twenty to thirty minutes starting with 0.1 c.c. and increasing the dose by 0.1 c.c. until symptoms are elicited or until sufficient is given. If symptoms develop one can attempt the repetition of smaller doses which had not caused symptoms. Where serum is to be administered intravenously, dilution and exceedingly slow administration at the beginning will help to avoid unfortunate results. Intraspinous injections should also be given slowly especially the initial portion in those comparatively recently injected.

The introduction of serum intravenously or intraspinously in those with the evidence of the extreme forms of hypersensitiveness should not be undertaken unless urgently required.

**Treatment of Developed Shock.**—Hypodermic injection of epinephrin or atropin will usually relieve the less severe attacks. In extreme collapse artificial respiration may be tried.

**Allergy to Foods, Pollens, etc.**—Rashes and other forms of reaction are commonly exhibited by a small percentage of individuals after the ingestion of a specific food. This may follow eggs, certain meats, fish, fruits, etc. The inducing substances may or may not be antigenic in character. Hay fever is an example of mucous membrane hypersensitiveness. Such individuals may show skin sensitiveness or develop rashes or other symptoms when the inciting substance is injected. Such hypersensitiveness may be toward pollens, dust from hair or skin of animals. The experimental work with pollen extracts seems conclusively to demonstrate that the inducing substances are not antigenic in character, viz., they do not stimulate antibody production nor will they sensitize experimental animals. Hay fever (and probably food allergy) is not due to sensitization of the individual but to an inherited predisposition. The hypersensitiveness to a specific agent is not necessarily inherited.

The repeated use of small doses or high dilutions of substances involved in these reactions, will develop an increased tolerance. This is only relative and is not comparable to the regularly induced and quantitatively greater resistance of desensitized animals.

Similar hypersensitiveness is shown in varieties of dermatitis due to poison-ivy, sumac, etc.

**Drug Allergy or Idiosyncrasy.**—Although toxic in larger amounts, the allergic symptoms follow a dose or doses, which are not appreciably toxic for most individuals. The symptoms elicited are evidently due to idiosyncrasy as they are different from those obtained with larger and uniformly toxic doses. The agents involved are various, including mercury, salvarsan, iodids, quinin, morphin, antipyrin, salicylic acid, turpentine, sandalwood oil, etc. The common symptoms are fever with or without chill, skin eruption, local edema or gangrene at the site of injection, swelling of the joints and lymph nodes.

As has been noted most of the phenomena of allergy to serums, etc., have much in common in their symptomatology. We have no evidence that the basis of these reactions is an antigen-antibody reaction, a hypothesis, which seems to be required in specific anaphylaxis. The dominant fact in human allergy is rather that of idiosyncrasy. In only a small number of cases has a condition been noted resembling anaphylaxis, viz., local necrosis after repeated injections, and symptoms of collapse after a second injection. These may have been actual anaphylactic reactions. We have no reason to believe that anaphylaxis could not be induced in man as in other animal species. To elicit actual anaphylactic shock, however, the dose would have to be relatively large, even if man were as easily sensitized and shocked as is the guinea-pig, which is extremely unlikely. The usual dosage in man is much lower per body weight than necessary to cause shock in the guinea-pig. In the case of intravenous or intraspinous injections we cannot ignore, however, the factor of quick absorption.

**Hypersensitiveness and Infection.**—In many infections there develops in variable degree a hypersensitiveness to the substance or products of the infecting type. The most marked examples of this are noted in infections due to the tubercle and to the glanders bacillus. Tuberculin which consists of the soluble products found in a broth culture of the tubercle bacillus will serve as an example for discussion.

**Tuberculin Hypersensitiveness.**—Tuberculin is only toxic for an infected animal, that is, infection results in hypersensitiveness. This hypersensitiveness is shown by the skin, the mucous membranes or by a systemic as well as by a focal reaction (site of lesion) when injected in sufficient doses. If the dose injected be sufficiently increased, death of the sensitive animal is caused. The substance or substances involved are highly resistant to heat, specific, but not anaphylactogenic. A relative tolerance can be induced in the tuberculous animal by gradually increased doses. The substance involved is different from all known anaphylactogenic substances and animals cannot be rendered hypersensitive by its injection. The proteins of the tubercle bacillus, however, are anaphylactogenic, but the hypersensitiveness to these, which can be induced in normal animals, is a distinctly different phenomenon. The mechanism of the tuberculin reaction is obscure. (See Tuberculosis.)

**Hypersensitiveness in Other Infections.**—A skin reaction is elicited in a considerable proportion of cases of syphilis by the intracutaneous injection of "luetin," an emulsion of the Treponema pallida. Similar indications of hypersensitiveness are noted in typhoid fever, or following the injection of typhoid vaccine by the ophthalmic or cutaneous method of introduction. Positive skin reactions have also been elicited by the gonococcus and also by some of the fungi. These reactions are relatively specific.

The practical value of the skin reactions other than that of tuberculin is materially reduced because of the relatively frequent occurrence of non-specific skin hypersensitiveness, which leads to confusing reactions. The generally advanced explanation is that a specific immune-body antigen complex is formed and this subjected to digestion by the local cells leads to the development of toxic products or to a neutralization of the antiferment and digestion of the host's proteins. As the reaction develops relatively slowly, such explanations may be warranted. The reactions described, however, may not be due in some instances at least directly to the protein *per se*. Some secretion product which we cannot identify may be involved. With our present knowledge we are not justified in assuming an identity in the nature of these reactions and that of tuberculin, the active principle of which is dialyzable, and apparently non-protein in nature. There is reason to believe, however, that the tuberculin in itself does not cause the reaction. The site of infection is much more sensitive to its action than are other areas. It would seem as if something were elaborated in the lesion which was a contributing agent. Many believe this to be an antibody and explain focal and the local reactions as due to a tuberculin plus antibody complex. We have little if any direct evidence for this assumption.

**Hypersensitiveness and Immunity.**—Jenner observed the more rapid appearance of a reaction following vaccination after a previous vaccinia. This observation has been studied by others and the evidence indicates that such an accelerated response is an indication of immunity. In the case of tuberculin, the reaction is also indicative of immunity to reinfection. The disappearance of the reaction in a person with a latent lesion, during measles for instance, is not infrequently followed by extension of the lesion and tuberculous disease. Gay attempted to show that the appearance of a positive skin reaction to typhoidin was an indication of immunity to typhoid fever. This at least is not absolute, as the reaction is absent or disappears at a time after infection or vaccination when immunity is known to still exist.

Because of the immunity-index character of the tuberculin reaction and the accelerated reactions observed in vaccinia, the conception that active immunity is basically a sensitization of the body cells has been advanced. This sensitization of the cells it is believed, is due to an acquired temporally and quantitatively exaggerated ability of response, the acquisition of this capacity being due to the stimulus of the immunizing agent. The basis of this ability we may conceive as due to the persistence of cellular antibodies (sessile). Stimulation may result also in the release of the antibodies as well as a rapid reproduction. In the case of antitoxic immunity this is actually demonstrable. In the case of the antimicrobal types of immunity the results of injections of vaccines in immune individuals indicates that the antibody response is greater in immune than in the normal individual. The results of some observers have shown striking differences. Other observers have obtained very much less marked results. Non-specific factors are involved to some extent in this difference. The injection of a non-specific vaccine according to Jobling will cause a dispersion or release of preformed antibodies, thus increasing the blood content. Gay attempted to show that immune typhoid rabbits responded with a specific leukocytosis, that is, a specific cell-production stimulation resulted when injected intravenously with typhoid vaccine. McWilliams was unable to verify these results.

## ENZYMES AND ANTI-ENZYMES.

**Microbal Enzymes.**—Pathogenic bacteria have enzymes which are necessary to their nutrition (see p. 52). When grown in media we find evidence of their action in proteolytic or carbohydrate cleavage or both. The degree of influence of such products in infection is not known. Certain bacteria secrete enzymes which are capable of digesting other bacteria such as pyocyanase secreted by the B. pyocyaneus. This product has been utilized for therapeutic purposes especially in localized infections.

**Enzymes (Ferments) of the Host.** Man and other animals possess digestive ferments not only in the intestinal canal but also in the body cells and fluids. As we have no evidence that proteins, microbal or other, are split by antibody-complement action we must look to these non-specific enzymes as the means by which the body disposes of parenterally introduced proteins. At least we are forced to this assumption unless we can demonstrate the development of specific enzymes for the introduced material. The apparent demonstration of this by Abderhalden is rendered very doubtful by the contradictory evidence of other observers. In some instances, the observations as to specificity of reaction seems conclusive, but this specificity may be due primarily to specific antibody action, the digestion being a secondary non-specific phenomenon. As we have seen under anaphylaxis the source of the digestion products may not come from the foreign protein but from the proteins of the host.

**Antienzymes (Antiferments) of the Host.**—These substances can be demonstrated in the blood serum and are apparently the means by which autodigestion is prevented. According to Jobling and Peterson the antitryptic action of blood serum is due to the lipoids. Under anaphylaxis we considered the hypothesis that these substances may have a direct bearing not only on the phenomenon of anaphylaxis but also on the development of disease symptoms. If Jobling's hypothesis be accepted the bacterial products alone or combined with their specific antibody will neutralize the antienzyme leading to the development of "serotoxin" by the action of the serum protease.

There is another phase which seems equally important. Bacteria seem to resist ferment action because of their lipoid content. Their limiting membrane is supposed to be lipoidal in character. This resistance is enhanced when bacteria are treated with lipoids. This would seem to explain both the resistance of the tubercle bacillus to digestion as well as the peculiar caseation of tuberculous lesions. The large amount of lipoids in the bacilli serves to inhibit the ferments liberated by disintegration of the tissue cells. The balance of enzyme and antienzyme is apparently a factor in the effects obtained in non-specific protein therapy (see under Vaccines).

**Aggressins—Virulins.**—Welch has offered the hypothesis, that bacteria like the cells of the host, react, with the production of protective antibodies which limit or prevent the action of the host's curative mechanism. Such adaptions would then underlie the characteristics of pathogenicity and virulence and explain the phenomena of bacterial resistance already described.

Bail believes the virulence of a bacterium depends on its ability to secrete "aggressins," hypothetical substances which prevent antibody action and paralyze the phagocytic cells. He believes that these substances are different from the toxic products of autolysis. Thus he found that if a bacterium was injected intraperitoneally into an animal, the resulting exudate after destruction of the contained bacteria by heat or chemicals and removal of the cells was relatively little if at all toxic, even in considerable amounts. If, however, this exudate was added to an amount of bacteria of the same type, which was not fatal in itself, the combination when injected would result in a rapidly fatal infection. This substance he considered different from the endotoxin. Wassermann and Citron, however, believe that the effect is due to the added action of the endotoxins in the exudate. Bail also claimed to have produced a specific immunity by injection of the "aggressins." Rosenau believes that an "aggressin-like substance" which he called "virulin" can be extracted from virulent pneumococci which will protect non-virulent pneumococci from opsonin action and therefore from phagocytosis and that non-virulent strains left in such extracts will acquire virulence. We have already mentioned the toxin-like cellular poison elaborated by some bacteria, viz., leukocidin.

**Antiblastic Immunity.**—This term was introduced by Ascoli to define the inhibitive action of immune serum on the metabolic processes of bacteria. Because of his failure to demonstrate a bactericidal or opsonic action *in vitro*, he believed that the activity of antianthrax serum resided in its antiblastic capacity, evidenced in one way by inhibition of capsule formation. Prior to his observation von Dungern and others observed similar inhibitive phenomena with immune sera, viz., restraint of pigment production by B. pyocyaneus, of proteolytic enzyme action by staphylococcus. In the latter case immune serum neutralized the enzymes obtained from the cocci indicating that an antienzyme antibody was involved. Dochez and Avery have more recently studied this phenomenon with antipneumococcus serum. Although antipneumococcus serum fails to cause bacteriolysis, it will inhibit multiplication for a certain period of time. This without further analysis is apparently due directly to antienzyme action or interference with the utilization of carbohydrates and nitrogenous compounds. A closer analysis (Blake[1]) indicates that the effect is due to agglutination, thus preventing the diffusion of the cocci throughout the medium and therefore diminishing the degree of utilization of foodstuffs. It is questionable how far the inhibition of special functions interferes with the infectious power of bacteria, especially when it is demonstrable that multiplication takes place in spite of such suppression.

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[1] Jour. Exper. Med., 1917, xxvi, 563.

# CHAPTER XIV.

# COMPLEMENT FIXATION: THE TECHNIC OF THE TEST AND ITS PRACTICAL APPLICATIONS.

In the previous chapter it was seen that cytolysis produced by the action of serum depends upon the presence within the serum of two components. These components are radically different in their properties; one is relatively heat resistant, or thermostabile, and is known as amboceptor; the other is easily destroyed by even ordinary temperatures (thermolabile) and aging, and is known as complement.

We have seen that amboceptor is a component of an immune serum, in which it may occur either as a result of an infection or of artificial immunization. Its production in the serum may be stimulated artificially by inoculating an animal with different antigens, such as microbal vaccines, protein extracts, or suspensions of foreign cells. The amboceptor produced by inoculation with any one antigen is specific for that antigen. This striking characteristic of amboceptor is seen in the strong combining affinity which exists between it and its antigen; thus typhoid amboceptor may be completely removed by saturating a typhoid-immune serum with a suspension of typhoid bacilli. In a similar way sheep erythrocytes will combine with their corresponding amboceptor in a hemolytic serum. When such a combination between an amboceptor and its antigen has taken place the resulting complex is fairly stable, but there is no visible change in the antigen. This fact is important, as we shall see later. An antigen thus combined with its amboceptor is said to be sensitized. Besides this property of specificity in combining with its antigen, amboceptor is further characterized by its resistance to heat; a serum containing amboceptor may be exposed repeatedly to a temperature of 56° C., with little or no loss in potency. This fact is utilized to obtain amboceptor free from complement from an immune serum. Thus it is seen that amboceptor is a component of immune serum, that it possesses a combining affinity for its specific antigen, and that it is comparatively heat resistant.

Complement, on the contrary, is a fragile component of all fresh serum, whether normal or immune. It disappears rapidly when the serum is exposed to moderate heat, and is therefore *thermolabile*, differing in this respect from amboceptor. It is preserved by low temperatures, and when frozen will easily maintain its potency for a week or longer. A serum which has lost its complement by heating or aging is no longer "fresh," and is said to be inactive. An inactive serum may be reactivated by adding to it a little fresh serum (complement). Complement is therefore non-specific; in itself it has no combining affinity for antigen; the amount of complement or its activity cannot be increased by artificial immunization.

We have seen then that amboceptor will combine with and sensitize its specific antigen, but that complement will not combine with either substance alone. We have seen also (Chapter XI) that when a sensitized antigen (if the antigen be cellular) is mixed with complement, lysis of the antigen results. Simultaneously a reaction affecting the complement occurs. This reaction was first described by Bordet and Gengou,[1] whose name it bears; as the sensitized antigen is being destroyed, complement apparently disappears from the mixture. This apparent disappearance is due to the *fixation* or *binding of complement*. It is not a visible phenomenon, and can be demonstrated only by indirect methods.

We have spoken previously of the destruction which results to the sensitized antigen when the latter is mixed with complement (Chapter XI). In the case of a bacterial antigen lysis is not easily demonstrable, but if we employ sensitized erythrocytes the resulting hemolysis may readily be observed by the passage of the hemoglobin from the cells to the surrounding fluid. It is for this reason that hemolysis is so frequently used to demonstrate serum cytolysis, of which it is only one type. The complex consisting of erythrocytes, their hemolytic amboceptor and complement is known as the hemolytic system. As in the case of bacteriolysis, hemolysis also results in the fixation of complement. The practical value of these different facts may now be seen by considering the following experiments of Bordet and Gengou.

Using a suspension of plague bacilli as antigen, inactivated antiplague horse serum as bacteriolytic amboceptor, and fresh guinea-pig serum as complement, six test-tubes were filled according to the following scheme.

1. Complement + plague bacilli + inactivated antiplague horse serum.
2. Complement + plague bacilli + inactivated normal horse serum.
3. Complement + inactivated antiplague horse serum.
4. Complement + inactivated normal horse serum.
5. Plague bacilli + inactivated anti-plague horse serum.
6. Plague bacilli + inactivated normal horse serum.

After the lapse of a certain time, rabbit erythrocytes and their specific hemolytic amboceptor were added to each tube. In the first, fifth and sixth tubes no hemolysis took place. In the second, third and fourth tubes hemolysis occurred. An analysis of these results may be made as follows: In the first tube we have a complete bacteriolytic system, composed of complement and sensitized bacterial antigen; failure to obtain hemolysis on the addition of the sensitized erythrocytes may be explained by assuming that complement had been fixed by the sensitized plague bacilli, and therefore was not "free" to complete the hemolytic system. No complement was placed in the fifth and sixth tubes, and we should therefore expect neither bacteriolysis nor hemolysis. In the second, third and fourth tubes at least one of the necessary constituents of a bacteriolytic system is missing; complement has not been "fixed," since on addition of sensitized erythrocytes hemolysis is obtained. We are therefore justified in concluding that in a specific or homologous antigen-antibody complex, complement is always fixed; and, further, that in the absence of either antigen or its specific amboceptor, fixation of complement cannot take place. It is now seen also how the use of sensitized erythrocytes may afford a test for the fixation of complement. A consideration of the following equations may help to make these facts clear:

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[1] Ann. de l'Inst. Past., 1901, xv, 290.

DIAGRAM OF POSITIVE COMPLEMENT FIXATION TEST

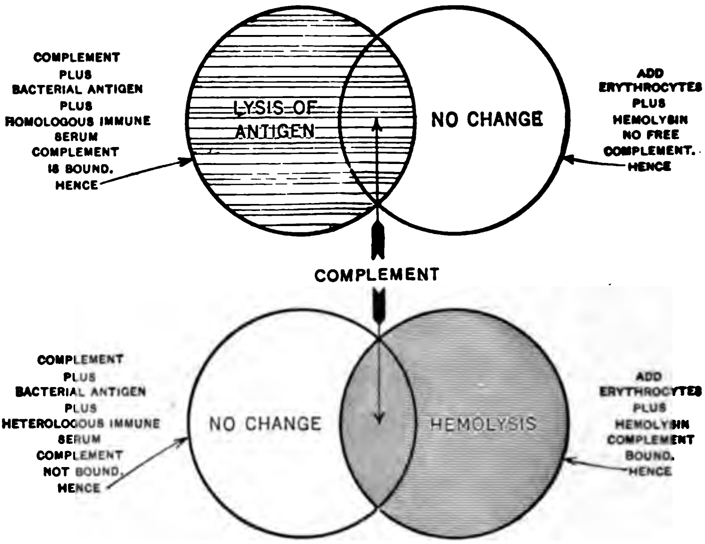
COMPLEMENT PLUS BACTERIAL ANTIGEN PLUS HOMOLOGOUS IMMUNE SERUM COMPLEMENT IS BOUND. HENCE
LYSIS OF ANTIGEN
NO CHANGE
ADD ERYTHROCYTES PLUS HEMOLYSIN NO FREE COMPLEMENT. HENCE
COMPLEMENT
COMPLEMENT PLUS BACTERIAL ANTIGEN PLUS HETEROLOGOUS IMMUNE SERUM COMPLEMENT NOT BOUND, HENCE
NO CHANGE
HEMOLYSIS
ADD ERYTHROCYTES PLUS HEMOLYSIN COMPLEMENT BOUND, HENCE

DIAGRAM OF NEGATIVE COMPLEMENT FIXATION TEST

FIG. 91

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| I. | Susceptible animal | + | Inoculation with antigen | | | = | Immune serum containing specific or homologous amboceptor (bacteriolytic, hemolytic, etc.) |
| II. | Bacterial antigen | + | Homologous bacteriolytic amboceptor | + | Complement | = | Fixation of complement (no visible reaction) |
| | | | **Bacteriolytic system** | | | | |
| III. | Bacterial antigen | + | Heterologous bacteriolytic amboceptor | + | Complement | = | No fixation of complement |
| IV. | Hemolytic antigen (erythrocytes) | + | Homologous hemolytic amboceptor | + | Complement | = | Fixation of complement, as shown by hemolysis |
| | | | **Hemolytic system** | | | | |

Having seen that complement can only be fixed when the system complex is complete, *i. e.*, when homologous[1] amboceptor and antigen are both present, the next step in the development and application of the phenomenon will be readily understood. Since we know that amboceptor is a component of immune serum and that it may be produced as a result of an infection we may assume conversely that the presence of a specific amboceptor in a serum may under certain conditions indicate that the host is or has recently been subject to an infection. This has already been more fully discussed in a previous chapter. Demonstration of the specific amboceptor in the serum can thus be made the basis of diagnostic tests. The presence of the amboceptor in the serum cannot be shown directly, but it may be tested for by means of complement-fixation. If in Bordet and Gengou's first experiment (for purpose of elucidation) inactivated serum from a patient had been used in place of the antiplague horse serum, and if on addition of sensitized erythrocytes no hemolysis had occurred, we should then have concluded that complement had been fixed by an antiplague amboceptor—antigen complex, since we have seen that an antigen can fix complement only when it has been sensitized by its amboceptor. The patient's serum must therefore have contained antiplague amboceptor; this would constitute *a positive test.* If, on the other hand, addition of the sensitized erythrocytes had resulted in hemolysis, absence of antiplague amboceptor from the patient's serum would have been indicated; this constitutes *a negative test.* Thus, by using known antigens, a serum may be tested for an unknown amboceptor, or an unknown antigen may be identified by testing it with known amboceptors. These facts are shown in the accompanying diagram:

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[1] This refers to the combination of antigen and its specific amboceptor. Other complexes than this can fix complement, one example has been noted in the precipitin reaction, another will be noted under the Wassermann reaction.

### DIAGRAMMATIC REPRESENTATION OF POSITIVE AND NEGATIVE COMPLEMENT FIXATION.

**I. Positive fixation**

| | | | | |
|---|---|---|---|---|
| Bacterial antigen<br>+<br>Homologous immune serum | + Complement<br>← | (Time interval allowed to elapse before addition of erythrocytes and hemolysin.) | + | Erythrocytes<br>+<br>Hemolytic amboceptor or hemolysin. |

**Complete bacteriolytic system, complement is fixed as shown by direction of arrow.**

Erythrocyte suspension remains cloudy, since complement has been fixed by bacteriolytic system and the cells remain intact.

**II. Negative fixation**

| | | | | |
|---|---|---|---|---|
| Bacterial antigen<br>+<br>Heterologous immune serum | + Complement<br>→ | (Time interval allowed to elapse before addition of erythrocytes and hemolysin.) | + | Erythrocytes<br>+<br>Hemolytic amboceptor. |

**Incomplete bacteriolytic system, complement remains free.**

Erythrocyte suspension is hemolized, since complement is free to combine with the sensitized erythrocytes, as shown by direction of arrow.

We have already seen (Chapter XI) that the complement-fixation test may be used (1) in diagnosing different infectious diseases, (2) as an aid in establishing the etiology of infectious diseases, (3) in the identification of proteins, and (4) in the standardization of immune sera.

The first practical application to the diagnosis of disease was attempted by Wassermann and Bruck[1] with the serum of tuberculous individuals. Their results were interesting but not of practical value. Their results a little later on attempting complement fixation with syphilitic serum were more encouraging. Testing the serum of monkeys which had been inoculated with human syphilitic material, with an antigen made by extracting human syphilitic organs fixation of complement was obtained. A short time later Wassermann, Neisser, Bruck and Schucht and others obtained similar results when testing human syphilitic serum. For this reason complement fixation as applied to the diagnosis of syphilis is known as the Wassermann reaction. This test is used very extensively. When it is properly carried out very accurate and useful information may be obtained. In order that a successful test may result, not only must all the ingredients be carefully prepared and properly used, but every part of the test must be safeguarded by carefully prepared controls.

## THE WASSERMANN REACTION.

**Technic.—Preparation and Standardization of Reagents.**—The reagents used in complement-fixation work are:

1. Salt solution.
2. Erythrocytes.
3. Hemolysin (homologous with the erythrocytes used).
4. Complement.
5. Immune serum (*i. e.*, patients' serum, serum containing unknown amboceptor, or bacteriolytic serum, hemolysin for standardization, etc.).
6. Antigen.

The *reaction* of the *reagents* and *glassware* has been found to have an important bearing on the accuracy of the test. *Acidity* and *alkalinity* may give rise to false reactions, which may be positive or negative according to the degree of acidity or alkalinity.

All *glassware*, especially new, should be neutralized after being cleaned. This may be accomplished by standing it in 1 per cent. hydrochloric acid solution overnight. It is then rinsed first in running tap water and then in hot distilled water. It may be dried and sterilized in the hot-air oven.

*Salt Solution* of *physiological strength* is used in performing the test, in diluting the different reagents and in washing erythrocytes. It should be isotonic with mammalian erythrocytes (0.85 per cent. to 0.9 per cent.) and should be tested for neutrality with phenolphthalein solution before being used. It may be prepared conveniently in large volumes (10 liters or more) at one time. It may be standardized after preparation (for percentage of sodium chloride) by the method recommended for chlorine determination in "Standard Methods for the Examination of Water and Sewage," American Public Health Association (1917), p. 42. Only chemically pure sodium chloride should be used.

*Erythrocytes* from sheep, goat, man, ox or other animal may be utilized in the hemolytic system. In our laboratory sheep erythrocytes are used after they have been washed six times and made up in a 5 per cent. suspension with salt solution.

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[1] Deutsch. med. Wchnschr., 1906, xxxii, 449.

The sheep are bled from the jugular vein into a sterile bottle or flask containing glass beads. Before the blood coagulates it is thoroughly shaken to defibrinate it. It is then filtered through two thicknesses of cheesecloth, to remove shreds of fibrin, and washed to obtain the erythrocytes free from serum (since the serum may give rise to anticomplementary reactions).

The erythrocytes are washed and prepared in the following manner: 2 to 4 c.c. of defibrinated blood are placed in a 15 c.c. graduated centrifuge tube and the tube is then filled with physiological salt solution. The defibrinated blood and salt solution are mixed by alternately drawing them into a pipette and expelling by means of a rubber bulb. The volume of salt solution should be at least three times that of the defibrinated blood, since otherwise the serum will not be completely removed. The mixture is centrifugalized rapidly for a sufficient time to throw the erythrocytes to the bottom of the tube. The supernatant fluid is decanted, or drawn off with the pipette, and replaced with fresh salt solution. The sedimented erythrocytes and saline are again mixed and centrifugalized. The process is repeated until the erythrocytes are entirely free from serum.[1] In many laboratories three washings are considered sufficient, but as we have found that even after four washings the serum is not always completely removed, we believe that better results will be obtained by *washing six times.* After the final washing the level of the erythrocytes is marked on the tube with a wax pencil before the cells have been disturbed by the removal of the supernatant fluid. The supernatant fluid is then discarded and the packed erythrocytes are diluted with salt solution to twenty times their volume. This gives us the 5 *per cent. suspension of erythrocytes.* The amount of defibrinated blood per centrifuge tube and the speed and duration of centrifugalization should always be the same, at least for the final washing, in order that the packing of the cells be uniform day by day.

Blood that has been withdrawn from the animal for more than two days in summer or three days in winter is usually unsuitable for hemolytic work, as the resistance of the erythrocytes becomes weaker and hemolysis occurs too rapidly, so that perfect balance of the hemolytic system cannot be obtained.

In places where it is inconvenient to obtain sheep cells, human erythrocytes are frequently used. An advantage in the human hemolytic system arises from the fact that by its use error in the test is avoided: human serum frequently contains natural antisheep amboceptor and the excess of hemolysin introduced into the hemolytic system when such a serum is being tested may, according to some investigators, cause a weakening or loss of a positive reaction. In our experience this error rarely or never occurs in the Wassermann reaction, owing to our practice of reading the reaction as soon as the controls of the test are completely hemolyzed (see section on Reading and Interpreting the Test, pp. 256 and 259); hence, we believe that the use of a hemolytic system other than sheep or the absorption of natural hemolysin from the serum is unnecessary in Wassermann work. In tests with bacterial antigens where the fixation of complement is less firm than in the Wassermann reaction the native antisheep hemolysin in human serum may be a source of error.

**Hemolysin.**—Hemolysin is obtained by successive inoculations of a rabbit (or other experimental animal) with the type of red blood cell to be used in tests. Inoculations may be made intraperitoneally or intravenously. Owing to the frequent occurrence of abscesses after intraperitoneal inoculations the intravenous method is preferable. In our laboratory, rabbits are inoculated intravenously at intervals of two days with fresh, thoroughly washed sheep erythrocytes in a 20 to a 50 per cent. suspension, the doses being 2 c.c., 4 c.c. and 6 c.c. On the ninth day after the last inoculation the rabbit is bled to death aseptically from the carotid or heart. The serum is placed in small sterile bottles or ampoules and heated for one-half hour at 56° C. on three successive days, to destroy the native complement and to ensure sterility. Hemolytic sera of high titer (1 to 3000) may be produced by this method.

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[1] The presence of serum may be determined by the nitric acid test.

*Standardization of Hemolysin.*—Hemolysin is standardized for the purpose of determining the unit. This is defined as the smallest amount of hemolysin which will give complete hemolysis of 0.1 c.c. of a 5 per cent. erythrocyte suspension, in the presence of an excess of complement, after an incubation period of one hour at 37° C. (water-bath). The standardization may be obtained by titration of one or more dilutions of the hemolytic immune serum, and for convenience, an attempt is made to determine that dilution of hemolysin in which 0.05 c.c. contains one unit.

The following table shows the scheme of titrating the hemolytic serum:

HEMOLYSIN TITRATION.[1]

| Number of tube. | Hemolytic serum in dilutions to be tested for the unit, c.c. | Ten per cent. complement. | Five per cent. erythrocyte suspension, c.c. | 0.85 per cent. salt solution, c.c. | |
|---|---|---|---|---|---|
| 1 | .1 | .1 | .1 | .1 | Incubated for one hour in the water-bath at 37° C. |
| 2 | .09 | .1 | .1 | .21 | |
| 3 | .08 | .1 | .1 | .22 | |
| 4 | .07 | .1 | .1 | .23 | |
| 5 | .06 | .1 | .1 | .24 | |
| 6 | .05 | .1 | .1 | .25 | |
| 7 | .04 | .1 | .1 | .26 | |
| 8 | .03 | .1 | .1 | .27 | |
| 9 | .02 | .1 | .1 | .28 | |
| 10 | .01 | .1 | .1 | .29 | |
| 11 | .1 | .0 | .1 | .3 | |
| 12 | .0 | .1 | .1 | .3 | |
| 13 | .0 | .0 | .1 | .4 | |

After thorough shaking the titration is incubated one hour in the water-bath at 37° C., and examined frequently to determine the rapidity of the reaction. As hemolysin is stable, and fresh erythrocytes from healthy sheep vary little in resistance to hemolysis such a titration indicates sufficiently for practical work the value of the complement. (See Standardization of Hemolytic System.)

In standardizing a new hemolytic immune serum (hemolysin) preliminary dilutions of 1 to 100, 1 to 1000, etc., are usually made, and titrated with fixed quantities of both the 5 per cent. erythrocyte suspension and the complement. It is necessary, however, that the amount of complement used be more than one unit.[2] This constitutes an "excess" of complement. After the titrations of the different dilutions have been incubated for one hour at 37° C. (water-bath), they are read to determine the smallest amount of hemolysin which gives complete hemolysis under the conditions imposed above, and from this reading that dilution in which the unit of hemolysin is contained in a volume of 0.05 c.c. may be determined by calculation.[3]

The interpretation of a hemolysin titration can be learned by experience only.

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[1] In the table above, the actual hemolysin titration is comprised in the first ten tubes. Tube 11 is a control of the hemolytic serum for lytic action without the aid of complement. Tube 12 is a control of the complement for lytic action without the aid of hemolysin. Tube 13 is a control of the stability of the erythrocyte suspension.

[2] The unit of complement is defined as the smallest amount of complement which will bring about complete hemolysis of 0.1 c.c. of 5 per cent. erythrocyte suspension sensitized by one unit of hemolysin, after incubation of one hour at 37° C. (water-bath). The unit of complement, therefore, depends on the previous determination of the unit of hemolysin.

[3] For example, if after titrating a dilution of 1 to 1000, tubes 1 to 8 inclusive (see table above) show complete hemolysis, while all tubes after 8 show partial or complete absence of hemolysis, then tube 8 will contain one unit of hemolysin. As the volume of the hemolysin dilution which was added to tube 8 was 0.03 c.c., then 0.03 c.c. of a dilution of 1 to 1000 is one hemolysin unit. As this volume (0.03 c.c.) is inconvenient to handle, we may calculate that dilution in which 0.05 c.c. is the unit as follows:

$$x : 1/1000 = 0.03 : 0.05$$

$$x = \frac{3}{5000}$$

$$\therefore x = \frac{1}{1600} \text{ (about)}$$

If a dilution of 1 to 1600 is now made and titrated we shall find that 0.05 c.c. will contain one hemolysin unit.

**Sensitizing the Erythrocyte Suspension.**—After hemolysin has been standardized it is usually customary to sensitize the erythrocytes for use in tests or for the standardization of other reagents. This may be done by adding two or three units of hemolysin to each 0.1 c.c. of 5 per cent. erythrocyte suspension. As will be seen later (standardization of the hemolytic system) a slight deficiency of complement may be replaced by increasing quantitatively the amount of hemolysin. During the "fixation period" in the actual test, complement, because of its nature, is deteriorating, and the rapidity of deterioration varies with the temperature. To ensure that the hemolytic system shall be sufficiently active at the end of a (negative) test, this deterioration of complement is provided for by sensitizing the unit volume of the erythrocyte suspension with at least two and sometimes three units of hemolysin, three units being employed only when the serum used for complement is weak in activating power. The danger of obtaining false positive reactions because of an inactive hemolytic system is thus avoided.

After the standardized hemolysin and the erythrocyte suspension have been thoroughly mixed in the proper amounts, they are placed on ice for a time until the erythrocytes are sensitized (usually one-half hour).

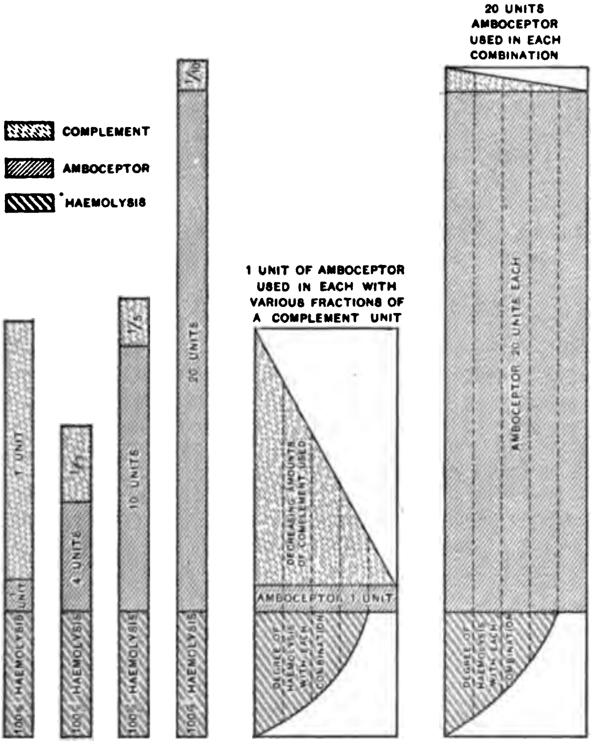
20 UNITS AMBOCEPTOR USED IN EACH COMBINATION
COMPLEMENT
AMBOCEPTOR
HAEMOLYSIS
1/10
20 UNITS
1/5
10 UNITS
1 UNIT
1 UNIT
1/2
4 UNITS
100% HAEMOLYSIS
100% HAEMOLYSIS
100% HAEMOLYSIS
100% HAEMOLYSIS
1 UNIT OF AMBOCEPTOR USED IN EACH WITH VARIOUS FRACTIONS OF A COMPLEMENT UNIT
DECREASING AMOUNTS OF COMPLEMENT USED
AMBOCEPTOR 1 UNIT
DEGREE OF HAEMOLYSIS WITH EACH COMBINATION
AMBOCEPTOR 20 UNITS EACH
DEGREE OF HAEMOLYSIS WITH EACH COMBINATION

**Fig. 92.**—Standardization of the hemolytic system. (Noguchi.)

**Standardization of the Hemolytic System.**—Complement fixation is, within limits, a quantitative reaction; that is, a sensitized antigen can fix only a limited amount of complement. Moreover, the extent or degree of sensitization of the antigen determines the amount of complement which it can fix. Thus in an actual complement-fixation test, two or more different amounts of the patient's serum are used with a constant amount of antigen, and the degree of fixation (*i. e.*, the amount of complement fixed) will vary with the different amounts of the patient's serum, the larger amounts of serum leading to the fixation of more complement (in case a positive serum was used).[1] An excess or a deficiency of complement in the test may therefore give rise to errors in diagnosis (since as stated above the amount of complement fixed depends on the degree of sensitization of the antigen). This is perhaps even more evident if we stop to consider the use in the test of the hemolytic system as an indicator of "free" complement. In the hemolytic system it can be shown that a deficiency of either complement or hemolysin may be replaced to a certain extent by increasing quantitatively the other; for example, if one "unit"[2] of hemolysin and one "unit" of complement acting together will cause complete lysis of a given quantity of erythrocytes, then one-tenth unit of complement may accomplish the same result, provided twenty units of hemolysin are used. This fact is clearly brought out in the accompanying diagram from Noguchi. Thus we see that for accurate results a careful determination should be made of the proper amount of complement to be used in the test; and also that an exact standardization of the hemolytic system is necessary. By such a standardization we mean the method by which the relative amounts of complement and hemolysin are determined, which together will hemolyze 0.1 c.c. of 5 per cent. erythrocyte suspension in one hour at 37° C. (water-bath).

Obviously such a standardization may be obtained by determining (by titration) both the hemolysin unit and the complement unit. Such a procedure is laborious, but in the case of complement fixation with bacterial antigens (*q. v.*) it is essential.[3] In the case of the Wassermann reaction where the fixation of complement is relatively firm it is usually sufficient to standardize the hemolytic system (1) by titrating the hemolysin (in which case a constant amount of complement (guinea-pig serum) is used in the tests, and the hemolysin varied), or (2) by titrating the complement (in which case a constant amount of hemolysin is used, and the complement varied).

Hemolysin, as we have seen, when once standardized is fairly stable, but the complement activity of the serum from different guinea-pigs varies widely. Hence, whatever method be employed a daily standardization of the hemolytic system must be made.

**Complement.**—Not all fresh serum has the power of reactivating the serum of an alien species or of being "fixed" by an antigen-amboceptor complex, but that of the guinea-pig has unusual power in reactivating human and rabbit sera; hence guinea-pig serum is generally used for complement in complement-fixation tests and in hemolytic work. There is wide variation in the activating power of the serum of individual guinea-pigs, and also in its "fixability" (*i. e.*, its power of combining with antigen and immune serum in the complement-fixation reaction). It is, therefore, advisable, if each pig's serum cannot be tested, to use the pooled serum of at least three guinea-pigs, in order that the complement may have average activating and combining properties. To be sure, however, of obtaining efficient complement for complement-fixation tests with bacterial antigens, it is essential that each pig's serum be tested for both activating power and fixability; all complements found by preliminary tests to be suitable are then pooled and titrated to determine the exact dose for use in tests

Guinea-pigs that have been used for antitoxin tests and other purposes may be of use in complement-fixation work after a rest of three months, but their serum is less liable than that of unused pigs to be of normal activating power. In the complement-fixation test for glanders, pigs that have been inoculated with horse serum should never be used, as substances are formed in their serum that cause a reaction with the serum of the horse that is being tested for glanders. In such an instance the serum controls of the test may in themselves inhibit hemolysis and no reading of the test can be made. Gravid pigs should not be used for complement, as their serum is liable to be weak in activating power.

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[1] This fact may be used as an arbitrary scale for reading and interpreting a complement-fixation test—see section on Reading and Interpreting the Test.

[2] The unit of hemolysin and the unit of complement have already been defined.

[3] The particular scheme by which the hemolytic system is standardized by the titration of both hemolysin and complement, as employed in our laboratory in complement-fixation tests with bacterial antigens, is given in the section on Complement Fixation with Antigens of the Gonococcus.

To obtain complement, guinea-pigs may be bled from the heart by aspiration and used again after several weeks' rest, or they may be bled to death. In our laboratory the pigs after being stunned by a blow at the base of the skull are bled from the throat[1] into Petri dishes or they may be bled to death from the heart after being chloroformed. In the latter case an incision is made into the thoracic cavity; the heart is grasped with forceps and cut into with sterile scissors. The blood is collected in sterile, neutral Petri dishes, left at room temperature protected from light for thirty minutes, then placed in the ice-box at 0° C. to 6° C. overnight. The serum is drawn off with a capillary pipette and centrifugalized to free it from blood cells. Before being pooled the serum from each pig should be tested separately for natural hemolysin, which is occasionally present, and for activating power. Serum containing natural hemolysin, or showing weak activating power should be discarded.

Complement deteriorates rapidly if exposed to sunlight or to a warm temperature (over 20° C.); if kept in the ice-box (at a temperature below 10° C.) it remains potent for at least twenty-four hours. Complement may be preserved for several weeks by freezing. Its activating power is thus retained, but its capacity for being bound may weaken so that it may not be reliable for use. In our experience complement frozen for one week is still perfectly satisfactory.

Complement is used in a 10 per cent. dilution made with physiological salt solution. (See Titration Table on p. 262.)

*Standardization of Complement.*—If an exact determination of the complement value or limit is desired, the hemolysin titration may be followed by a complement titration. (See Section on Standardization of Hemolytic System by Complement Titration, p. 264.)

For Preparation and Standardization of Antigen see p. 268.

**Technic of Complement-fixation Test for Diagnosis.**—A test for diagnosis is really a simplified antibody content titration (*q. v.*) of a patient's serum with a known antigen; the nature of the amboceptor and the amount present both being unknown.

There are a number of different schemes for conducting tests for diagnosis; most of these have been developed from Bordet and Gengou's original method and from Wassermann's modification as applied to the diagnosis of syphilis, especially the latter. Because of its fundamental importance, Wassermann's original scheme is given below.

CLASSICAL WASSERMANN TEST.

| Number of tube. | Luetic liver extract diluted, c.c. | Patient's serum, c.c. | Ten per cent. complement, c.c. | 0.85 per cent. saline, c.c. | | Sensitized erythrocyte suspension, c.c. | |
|---|---|---|---|---|---|---|---|
| 1 | 1.0 | 0.2 | 1.0 | 0.8 | Incubated one hour | 2.0 | Incubated until control tubes are completely hemolyzed. |
| 2 | 0.5 | 0.1 | 1.0 | 1.4 | | 2.0 | |
| 3 | 2.0 | 0.2 | 1.0 | 1.8 | | 2.0 | |
| 4 | 2.0 | 0.0 | 1.0 | 0.0 | | 2.0 | |

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[1] Care must be taken not to cut the esophagus, as the stomach contents may thereby be mixed with the serum. If this occurs, the serum is unfit for use in complement-fixation work.

Our technic differs in three respects from that of Wassermann: (1) in the volume of the test, (2) in the amount of antigen used, and (3) in the method of fixation. It will be seen in what follows that the particular scheme which we ourselves use depends on the particular diagnosis being made; thus tests of serum for syphilis, with lipoidal antigens, are carried out according to one scheme, tests of spinal fluid (for syphilis), according to a second scheme, while tests with bacterial antigens require an entirely different arrangement. These different schemes are tabulated below.

TEST OF SERUM FOR DIAGNOSIS OF SYPHILIS.

| Number of tube. | Patient's serum, c.c. | Antigen in standard dilution, c.c. | Ten per cent. complement, c.c. | 0.85 per cent. saline, c.c. | | Sensitized erythrocyte suspension, c.c. | |
|---|---|---|---|---|---|---|---|
| 1 | .02 | .1 | .1 | .1 | Period for fixation[1] | .2 | Incubated until hemolysis is complete in tubes 3 to 6 |
| 2 | .01 | .1 | .1 | .1 | | .2 | |
| 3 | .04 | .0 | .1 | .2 | | .2 | |
| 4 | .02 | .0 | .1 | .2 | | .2 | |
| 5 | .0 | .2 | .1 | .0 | | .2 | |
| 6 | .0 | .1 | .1 | .1 | | .2 | |

Serum controls
1. Duplicate diagnostic test to detect error in technic.
2. Test for natural antisheep amboceptor to avoid false negative reactions.
3. Test for anticomplementary unit.
4. Test for lytic action upon cells alone.

Antigen controls
1. For specificity of antigen.
   - Diagnostic test with known positive human serum.
   - Diagnostic test with known negative human serum.
2. Test for anticomplementary unit.
3. Test for fixation unit.
4. Test for lytic action with complement when no amboceptor is used.

Control tests of each serum and of the antigen are made as in the classical Wassermann.

A test of serum for the diagnosis of syphilis is arranged as in the table above. The reagents should all be prepared and standardized, and the patient's serum inactivated, as described in the previous sections, before the test is undertaken.

TEST OF SPINAL FLUID FOR DIAGNOSIS OF SYPHILIS.

| Number of tube. | Patient's spinal fluid, c.c. | Antigen in standard dilution, c.c. | Ten per cent. complement, c.c. | 0.85 per cent. saline, c.c. | | Sensitised erythrocyte suspension, c.c. | |
|---|---|---|---|---|---|---|---|
| 1 | .2 | .1 | .1 | .1 | Period for fixation (4 hours in ice-box at 8° C.) | .25 | Incubated (water bath 37.5° C.) until hemolysis is complete in tubes 5 and 6. |
| 2 | .1 | .1 | .1 | .1 | | .25 | |
| 3 | .2 | .1 | .1 | .1 | | .25 | |
| 4 | .1 | .1 | .1 | .1 | | .25 | |
| 5 | .2 | 0 | .1 | 0 | | .25 | |
| 6 | .4 | 0 | .1 | 0 | | .25 | |
| 7 | .08 | .1 | .1 | .2 | | .25 | |
| 8 | .06 | .1 | .1 | .2 | | .25 | |
| 9 | .04 | .1 | .1 | .2 | | .25 | |
| 10 | .02 | .1 | .1 | .2 | | .25 | |
| 11 | .01 | .1 | .1 | .2 | | .25 | |

**N. B.—Spinal fluid is always used without being inactivated.**

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[4] Four hours in ice-box if crude alcoholic extract "antigen" is used.

In our experience the results obtained by using all the reagents in one-tenth the amounts used by Wassermann are absolutely reliable, provided the test is accurately performed. The advantages of the small volume of our test are in the saving of material (this is considerable) and in the very small amount (about 0.2 c.c.) of the patient's serum which is sufficient for the test. We have found that the use of a constant amount of antigen gives essentially the same results as the use of varying amounts.

TEST OF SERUM FOR DIAGNOSIS WITH BACTERIAL ANTIGENS, SETTING UP THE DIAGNOSTIC TEST IN DUPLICATE.

| Tube No. | | Patient's serum undiluted, c.c. | Antigen standard dilution, c.c. | Complement standard dilution, c.c. | Saline solution .9 per cent. | | Sensitized cells. | Five per cent. suspension. |
|---|---|---|---|---|---|---|---|---|
| Serum a/c control | 1 | .04 | .0 | .1 | .06 | Fix 6 hours ice-box at 8° C. or 1 hour in water bath at 37° C., and then add sensitized cells. | .2 | .0 |
| | 2 | .02 | .0 | .1 | .08 | | .2 | 0 |
| Diagnosis test in duplicate | 3 | .02 | .1 | .1 | .08 | | .2 | 0 |
| | 4 | .01 | .1 | .1 | .09 | | .2 | 0 |
| | 5 | .02 | .1 | .1 | .08 | | .2 | 0 |
| | 6 | .01 | .1 | .1 | .09 | | .2 | 0 |
| Antigen a/c control | 7 | 0 | .4 | .1 | 0 | | .2 | 0 |
| | 8 | 0 | .3 | .1 | 0 | | .2 | 0 |
| | 9 | 0 | .2 | .1 | 0 | | .2 | 0 |
| | 10 | 0 | .1 | .1 | .1 | | .2 | 0 |
| System control | 11 | 0 | 0 | .1 | .2 | | .2 | 0 |
| Cell control | 12 | 0 | 0 | 0 | .3 | | .2 | 0 |
| Natural-antisheep amboceptor control | 13 | .04 | 0 | .1 | .16 | | .0 | .1 |
| | 14 | .02 | 0 | .1 | .18 | | 0 | .1 |
| | 15 | .02 | .1 | .1 | .18 | | 0 | .1 |
| | 16 | .01 | .1 | .1 | .19 | | 0 | .1 |

In these tables "a/c" means anticomplementary.

**The Wassermann Reaction.**—As we have stated in a previous section the complement-fixation test as applied to the diagnosis of syphilis is known as the Wassermann reaction.

The antigen originally employed by Wassermann, Neisser and Bruck consisted of a saline extract of liver from a syphilitic fetus. The finely divided tissues were mixed in the proportion of 1 to 4, with physiological salt solution, to which 0.5 per cent. phenol had been added, agitated at room temperature for twenty-four hours and centrifugalized. The supernatant fluid was used as the antigen. Since the cultivation of the Treponema pallidum has become possible, antigens have been made from the organisms in pure culture instead of from luetic tissue; but the results have, on the whole, been less satisfactory, *i. e.*, fewer cases of syphilis give a positive Wassermann reaction with a Treponema pallidum antigen than with luetic tissue antigen or with a lipoid antigen from normal tissues.

The rationale of the reaction was at first supposed to be the same as that of Bordet and Gengou's first experiment (see p. 245); that is, a reaction between the specific antigen (extract of Treponema pallidum from the syphilitic organ) and an antisyphilitic amboceptor in the human serum. This view became untenable when complement fixation was soon obtained by different workers, with syphilitic serum and antigens prepared by extracting normal organs. A conception of the reaction as due to a specific immune complex of antigen and antibody is therefore no longer possible. As we have already seen, these different antigens consist largely of lipoids (oleates, lecithins and cholesterol) and do not appear to bear any relationship to the true antigenic substance which stimulates the production of the complement-fixing bodies (called by Citron "Luesreagine") in syphilitic serum. Inoculation of these lipoidal antigens will not stimulate the production of complement-fixing amboceptors in the serum of experimental animals. Nevertheless, they react *in vitro* with the Luesreagine in syphilitic serum. Also in syphilitic serum the globulin fraction is increased. It is possible that Citron's Luesreagine is identical with this globulin fraction. It has been suggested that the colloidal lipoids of these antigens combine with and precipitate the globulin of the syphilitic serum and that the resulting precipitate adsorbs complement. Whatever be the true explanation of the phenomenon we know only that the reaction is a lipotropic or lipoidophilic one. (Noguchi.)

INTERPRETATION OF COMPLEMENT-FIXATION REACTIONS IN TEST AND CONTROLS.

| Tube No. | Purpose of test. | A Reactions indicating a true positive. | B Reactions indicating a true negative. | C Reactions indicating a false positive. | **D Reactions indicating a false negative.** |
|---|---|---|---|---|---|
| 1 | Control of a/c reaction | Completely hemolyzed | Completely hemolyzed | Completely fixed | **Completely hemolyzed.** |
| 2 | Of patient's serum | Completely hemolyzed | Completely hemolyzed | Completely fixed | **Completely hemolyzed.** |
| 3 | Original test | Completely fixed | Completely hemolyzed | Completely fixed | **Completely hemolyzed.** |
| 4 | Original test | Completely fixed | Completely hemolyzed | Completely fixed | **Completely hemolyzed.** |
| 5 | Duplicate test | Completely fixed | Completely hemolyzed | Completely fixed | **Completely hemolyzed** |
| 6 | Duplicate test | Completely fixed | Completely hemolyzed | Completely fixed | **Completely hemolyzed.** |
| 7 | Control of a/c reaction of antigen | Trace of fixation | Trace of fixation | Trace of fixation | Trace of fixation. |
| 8 | | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed. |
| 9 | | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed. |
| 10 | | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed | Completely hemolyzed. |
| 11 | Control of complement activity | Hemolyzed in 10 minutes | Hemolyzed in 10 minutes | Hemolyzed in 10 minutes | Hemolyzed in 10 minutes. |
| 12 | Stability of cells; reading control | No hemolysis | No hemolysis | No hemolysis | No hemolysis. |
| 13 | Control for natural antisheep amboceptor in patient's serum | No hemolysis | No hemolysis | No hemolysis | Completely hemolyzed. |
| 14 | | No hemolysis | No hemolysis | No hemolysis | Completely hemolyzed. |
| 15 | | No hemolysis | No hemolysis | No hemolysis | Completely fixed. |
| 16 | | No hemolysis | No hemolysis | No hemolysis | Completely fixed. |
| Complement-fixation diagnosis | | 4 + | Negative | No diagnosis. Patient's serum is anticomplementary | 4 + Natural amboceptor in patient's serum interfered with fixation test. |

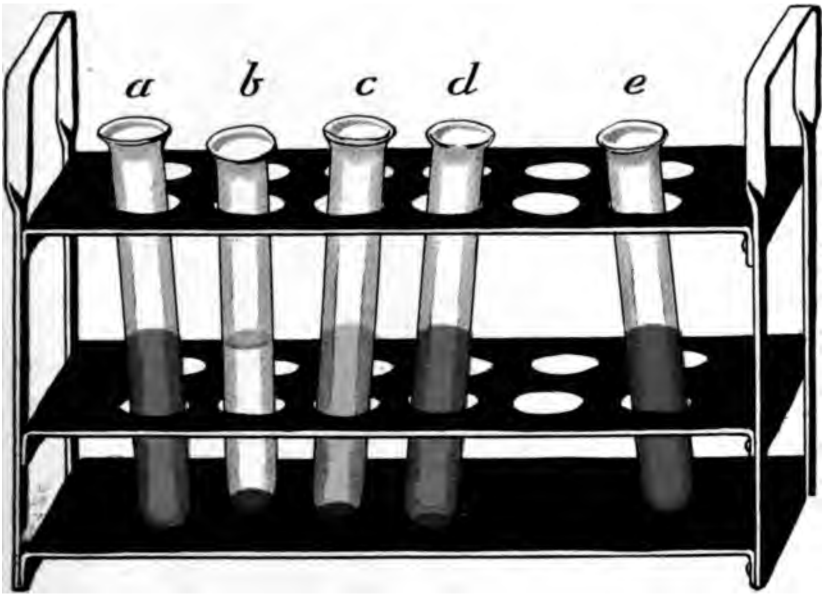
Column (A) represents the reactions which are interpreted as a 4 +.

Column (B) represents the reactions which are interpreted as a negative.

Column (C) represents false positive reaction in test due to an anticomplementary patient's serum.

Colum (D) represents a false negative (occurring rarely) due to an excess of amboceptor. Tubes (13) and (14) contain same reagents as tubes (1) and (2); tubes (15) and (16) same as (3) and (4), except that after the fixation period no artificial amboceptor is added to (13), (14), (15) and (16). Therefore the hemolysis in (13) and (14) is due to natural antisheep amboceptor in patient's serum. A true 4+ diagnosis can be made upon the control (13), (14), (15), (16).

## PLATE V



*a.* Complete absence of hemolysis before settling of cells.
*b.* Complete absence of hemolysis after settling of cells.
*c.* Partial hemolysis.
*d.* Nearly complete hemolysis.
*e.* Complete hemolysis.

Though biologically non-specific, the Wassermann reaction is clinically specific except, perhaps, when certain methods are employed in cases of leprosy, yaws, sleeping sickness and scarlet fever.[1] With these exceptions a positive reaction indicates the presence of luetic infection, either active or latent. A positive reaction may be given in any stage of syphilis, but is most liable to occur in the secondary stage. A negative reaction at any stage of the disease does not exclude the possibility of syphilis. Antiluetic treatment, especially mercury, frequently results in the reaction becoming negative, though the disease may still be active.

Treatment with salvarsan and even with mercury and the iodides may at first or after a few treatments cause a negative Wassermann to become positive for a time. A cure can only be pronounced when the reaction has remained negative for at least a year after intensive treatment and when it then still remains negative after a provocative treatment with salvarsan.

The ingestion of alcohol or the administration of an anesthetic within twenty-four hours before the collection of the blood specimen may interfere with the accuracy of the test, alcohol weakening the reaction and an anesthetic giving rise to false positive reactions.

Spinal fluid may give a positive reaction when the blood serum is negative in cases in which the brain or cord is involved. Hence the spinal fluid should always be tested in addition to the serum, if that is negative, whenever syphilis of the nervous system is suspected.

The addition of cholesterol to the extent of half-saturation to a crude alcoholic extract antigen has been recommended by some serologists; such an antigen gives a higher percentage of positive reactions than the crude extracts, but its reliability has not yet been established, at least for primary diagnosis.

In our experience a cholesterinized antigen is of special value in determining the effectiveness of antiluetic treatment, as old cases of syphilis that are or have recently been under treatment are more liable to react with this antigen than with a crude alcoholic antigen. In making a fresh diagnosis it is not wise to depend on a cholesterinized antigen alone, since cases of scarlet fever, leprosy and other non-syphilitic conditions have been found to react strongly with this antigen. These false positive reactions do not occur with a crude alcoholic antigen. A positive reaction is, however, presumptive of syphilis; a negative reaction with the cholesterinized antigen has therefore greater value in excluding a diagnosis of syphilis than has a negative reaction with the crude alcoholic antigen.

**Controls.**--Each series of diagnostic tests should be accompanied by controls of (1) the fixability of the antigen, (2) the specificity of the antigen, and (3) the anticomplementary and lytic doses of the antigen. We believe also that it is advisable to include with the tests of the day a titration of the antigen, using the same method of fixation as is employed in the tests. (See below, Period of Fixation.)

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[1] With the use of the crude alcoholic antigens, in one-quarter of the anticomplementary dose, and ice-box fixation, we have never obtained false reactions in such cases.

SCHEME FOR THE ARRANGEMENT OF THE ANTIGEN CONTROLS.

| Number of tube. | Positive (++++) serum, c.c. | Negative serum, c.c. | Antigen in standard dilution, c.c. | Ten per cent. complement, c.c. | 0.85 per cent. saline, c.c. | | Sensitized erythrocyte suspension, c.c. | |
|---|---|---|---|---|---|---|---|---|
| 1 | .02 | .0 | .1 | .1 | .1 | Period of fixation same as for the test. | .2 | Incubated until hemolysis is complete in tubes 3 to 12, inclusive. |
| 2 | .01 | .0 | .1 | .1 | .1 | | .2 | |
| 3 | .0 | .02 | .1 | .1 | .1 | | .2 | |
| 4 | .0 | .01 | .1 | .1 | .1 | | .2 | |
| 5 | .0 | .0 | .2 | .1 | .0 | | .2 | |
| 6 | .0 | .0 | .1 | .1 | .1 | | .2 | |
| 7 | .04 | .0 | .0 | .1 | .2 | | .2 | |
| 8 | .02 | .0 | .0 | .1 | .2 | | .2 | |
| 9 | .0 | .04 | .0 | .1 | .2 | | .2 | |
| 10 | .0 | .02 | .0 | .1 | .2 | | .2 | |
| 11 | .0 | .0 | .2 | .1 | .0 | | .2 | |
| 12 | .0 | .0 | .1 | .1 | .1 | | .2 | |

Tubes 1 and 2 are positive controls, or controls of the fixability of the antigen.
Tubes 3 and 4 are negative controls, or controls of the specificity of the antigen.
Tubes 5, 6, 11 and 12 are controls of the anticomplementary and lytic properties of antigen.
Tubes 7, 8, 9 and 10 are controls of the anticomplementary properties of the positive and negative sera.

**Period of Fixation.**—In the original Wassermann test a time interval of one hour was allowed for the fixation of complement before the addition of hemolysin and erythrocytes and the temperature of fixation was 37° C. (incubator). While the reason is not perfectly understood, it has since been found (McNeil in our own laboratory) that the complement-fixation reaction is more delicate when the mixture of the patient's serum, antigen and complement are allowed to combine at ice-box temperature (8° C.).[1] Since the velocity of fixation varies directly with the temperature (up to 37° C.), the fixation time must be proportionately increased if the lower temperature is used. It is probable that with the longer exposure at the low temperature a firmer and more complete fixation of complement results than is possible with the shorter period at incubator temperature, while it is unwise to lengthen the fixation period in the latter case because of the more rapid deterioration of complement.

With bacterial antigens ice-box fixation has given us our most satisfactory results.[2] The exact time of fixation varies, however, with antigens of the different bacteria, and is therefore given in the section on "Complement Fixation with Bacterial Antigens," under the different types of bacteria considered.

In the diagnosis of syphilis also, with lipoidal antigens, the period of fixation varies with the different antigens used. With the crude alcoholic extract antigens we are accustomed to use a fixation period of four hours in the ice-box at 8° C., but with cholesterinized antigens we believe that more reliable diagnosis is possible with a fixation period of one hour in the incubator at 37° C. With Noguchi's acetone insoluble fraction antigen, fixation is for one hour in the incubator at 37° C.

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[1] It is our experience that room temperature (20° C.) is not desirable for fixation, as complement deteriorates too rapidly during the prolonged time interval required.

[2] Ten per cent. more positive cases are obtained by this method than if incubator fixation is used.

**Reading and Interpreting the Test.**—After the period of fixation is completed a standard amount (indicated in the different tables) of sensitized erythrocytes is added to all tubes. The tests are then placed in a water-bath at 37° C. until the antigen control and serum control tubes show complete hemolysis at the end of eight minutes. Readings of the degree of complement fixation in each tube of the test are then made. We are accustomed to using Citron's[1] standard in reading all tests. (Plate V.)

Complete absence of hemolysis in tubes 1 and 2 = ++++, very strong positive.

Complete absence of hemolysis in tube 1, faint hemolysis in tube 2 = +++, strong positive.

Complete absence of hemolysis in tube 1, complete or nearly complete hemolysis in tube 2 = ++, positive.

Partial hemolysis in tube 1, complete or nearly complete hemolysis in tube 2 = +, weak positive or doubtful.

Nearly complete hemolysis in tube 1, complete hemolysis in tube 2 = ±; doubtful.

Complete hemolysis in all tubes = −, negative.

The distinction between ++ and + is very important, as the latter reaction is said to be frequently non-specific. We therefore make a positive diagnosis on only ++, +++, or ++++ reaction.[2]

**Reading and Interpreting Tests on Spinal Fluid.**—In testing spinal fluid for syphilis it is customary to use relatively large amounts (ten times as much fluid as serum). (See Table above.)

Because of the large number of the different amounts of spinal fluid used in the test, a reading and interpretation according to Citron's standard is not possible. As most spinal fluids are from patients who have already been diagnosed as syphilitic, complete fixation of complement by antigen and any amount of spinal fluid (as used above) is considered a positive test; partial fixation by antigen and any of the larger amounts of spinal fluid is considered a doubtful reaction; while complete hemolysis in all amounts of fluid is of course a negative reaction. If a series of tests similar to the above be carried out on spinal fluid withdrawn at different intervals from a patient over a prolonged period of time, and if the exact amount of inhibition of hemolysis in each tube be recorded in each test and compared with the degree of inhibition in the corresponding tubes in the succeeding tests of the series, such a scheme may be of value in observing and following the course of treatment.

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[1] Citron's Immunity, translated by Garbat.

[2] A + Wassermann reaction following the treatment of a case that has given a ++, +++ or ++++ reaction is considered positive and indicates the need of further treatment.

If a reading according to Citron's standard is desired, spinal fluid may be tested according to the scheme for testing serum; but in this case the amounts of spinal fluid should be double the amounts of serum.

**The Complement-fixation Test for Gonococcus.**—The Bordet-Gengou[1] reaction was applied to the diagnosis of gonococcus infections by Müller and Oppenheim[2] in 1906, and in the same year Brück[3] working with Wassermann made a comparative study of the fixation and agglutination reactions. Later (1907) Teague and Torrey,[4] and Watabiki,[5] attempted to group all gonococci by the complement-fixation test.

Schwartz and McNeil[6] (1911) made a comprehensive study of the reaction in relation to the clinical diagnosis and drew decisive conclusions favorable to the value of the test. Their technic has been widely followed and many later workers[7] [8] [9] [10] [11] [12] [13] have corroborated their results. Schwartz and McNeil used a polyvalent antigen made from the ten strains identified by Torrey. This was an aqueous extract of living gonococci. Other kinds of antigen have been described by later workers, notably Warden's "specific fats" and Thomson's dissolved whole cocci. Wilson of this laboratory, working with McNeil in 1916, developed the defatted antigen described on page 270, and since then this lipoid-free product has been used in many of the New York laboratories, and in several other cities throughout the country. The advantage of the defatted antigen is its stability, freedom from anticomplementary reaction and comparatively high titer. The aqueous products were always used in low dilution and would deteriorate in a few weeks or months, while the heated suspensions of defatted cocci have an average titer of 1 to 20 dilution and are perfectly stable, retaining their antigenicity for more than a year. This is a practical advantage, as it enables one to make up a large stock at one time, thus reducing the cost of production. As regards the value of the defatted antigen in "picking up" more positive reactions in cases having a small amount of the gonococcus antibody, a comparison of our results with the publications of workers using various other kinds of antigen show practically the same percentage of positives in the same clinical types of cases, and would indicate that the antigenic property is not confined to any particular part of the coccus body. The reports of studies on gonococcus complement fixation show an agreement of opinion on the following points:

Cases of anterior gonorrheal urethritis and acute vulvovaginitis rarely give a positive complement-fixation test.

[1] Ann. de l'Inst. Pasteur, 1907, xv, 290.

[2] Wiener klin. Wchnschr., 1906, xix, 894.

[3] Deutsch. med. Wchnschr., 1906, xxxiii, No. 34, 945; xliv, 10 and 110; ix, 588.

[4] Jour. Med. Res., n. s., xii, 236.

[5] Jour. Med. Res., 1909, 20, 365.

[6] Am. Jour. Med. Sc., cxliv, 815.

[7] Swinburne: Arch. Diag., July, 1911.

[8] Keyes: Am. Jour. Med. Sc., January, 1912.

[9] Schmidt: Tr. Am. Urol. Assn., 1911.

[10] Gradwohl: Am. Jour. Derm. and Syph. Urin. Dis., June, 1912.

[11] Kolmer: Text-book of Infect. Im. and Spec. Ther., ed. 1915, p. 482.

[12] Warden: Jour. Infect. Dis., 1915, xvi, 426.

[13] Thomson: Lancet, July 13, 1918, p. 42.

A positive reaction is indicative of the presence, or recent activity, in the body of a focus of living gonococci.

A negative reaction does not exclude gonococcus infection.

A strong positive reaction is not to be expected before the fourth week. A positive reaction does not disappear entirely until seven or eight weeks after cure.

Persistently negative results obtained through a considerable period of time indicate the probability of a cure.

The gonococcus complement-fixation test is specific as regards human serums. We have made specificity tests upon a large number of human serums and spinal fluids, from cases of syphilis, tuberculosis, meningitis and poliomyelitis, and obtained negative reactions in all, except a few of the syphilitic serums which had a coincident history of gonorrhea.

**Technic.**—Complement; Guinea-pig's serum, prepared as described on page 250. The earlier writers recommended the use of a pool of several guinea-pig serums in order to avoid variation in the complements used from day to day, although they were all agreed that there was no specificity of complement. In our titration of immune serums we found that there is considerable specificity for fixability among guinea-pig serums,[1] as well as the variation in hemolytic activity and anticomplementary properties recognized by all workers. The latter variants can be controlled by dilution of the complement, but if the serum is lacking in fixability it should be excluded from the pool, as it may weaken, or render negative, the value of the whole pool. The hemolytic and fixation strengths of a serum are not always proportionate, that is, one serum may have a low hemolytic titer and give a weak or negative fixation reaction. On the other hand, a serum of high hemolytic activity will often be very strongly fixed by the antigen-antibody combination. This type of serum has given us some of our highest titers of immune serums. To obtain an efficient complement for bacterial antigen fixation, it is essential, we repeat, that every pig's serum be tested before pooling. This preliminary test establishes the value of the complement from each pig, and does away with the necessity for pooling in order to avoid variations. This is of practical value to the small laboratory where the number of tests does not require a large volume of complement. The good complement from one pig is just as efficient as the good complement from ten pigs, and much better than a pool of good and bad serums.

*Method for Making Preliminary Tests before Pooling.*—First, bleed the guinea-pigs and prepare the serum as described on page 253.

Second, dilute the serum from each pig 1 to 10 with 0.9 per cent. saline.

Third, test the 1 to 10 dilution of each pig's serum for the presence of natural antisheep amboceptor and for its hemolytic activity. These tests are set up as follows:

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[1] About 60 per cent. of all pigs bled during the past two years could be used for gonococcus fixation.

| Test No. | Purpose of test. | | Guinea-pig serum, 1 to 10 dilution, c.c. | 0.9 per cent. saline, c.c. | 5 per cent. suspension c.c. | Sensitized cells.[1] |
|---|---|---|---|---|---|---|
| 1 | Test for natural antisheep amboceptor | | .2 | .2 | .1 | 0 |
| 2 | Test for hemolytic activity | Tube No. 1 | .1 | 0 | 0 | .2 |
| | | 2 | .09 | .01 | 0 | .2 |
| | | 3 | .08 | .02 | 0 | .2 |
| | | 4 | .07 | .03 | 0 | .2 |
| | | 5 | .06 | .04 | 0 | .2 |
| | | 6 | .05 | .05 | 0 | .2 |
| | | 7 | .04 | .06 | 0 | .2 |
| | | 8 | .03 | .07 | 0 | .2 |
| | | 9 | .02 | .08 | 0 | .2 |
| | | 10 | .01 | .09 | 0 | .2 |

Place these tests in the water-bath at 37° C. for thirty minutes. At that time the number (1) test of each pig should show no hemolysis. If it does it indicates the presence of natural antisheep amboceptor in the guinea-pig's serum.

In No. 2 test read the unit of hemolysis fot each pig, then for all those complements of which the unit of hemolysis is other than 0.05 c.c., make a new dilution in which 0.1 c.c. of the new dilution will contain two hemolytic units of the complement.[2]

| Test No. | Purpose of test. | Antigen standard dilution. c.c. | Serum. c.c. | Guinea-pig serum 2-unit dilution. c.c. | 0.9 per cent. saline. c.c. | | Sensitized cells. c.c. |
|---|---|---|---|---|---|---|---|
| 3 | Anticomplementary with antigen | .4 | 0 | .1 | .. | After fixation period, add sensitized cells | .2 |
| | | .3 | 0 | .1 | .. | | .2 |
| | | .2 | 0 | .1 | . | | .2 |
| | | .1 | 0 | .1 | .1 | | .2 |
| 4 | Anticomplementary with serum, a known positive serum, undiluted | 0 | .04 | .1 | .2 | | .2 |
| 5 | Fixability | .1 | .01 | .1 | .1 | | .2 |
| Controls: | | | | | | | |
| 6 | Complement stability | 0 | 0 | .1 | .2 | | .2 |
| 7 | Sensitized cell stability | 0 | 0 | 0 | .3 | | .2 |

---

[1] This dose of sensitized cells contains 0.1 c.c. of 5 per cent. suspension of sheep cells plus 0.1 c.c. of the standard dilution of hemolysin.

[2] If the unit of hemolysis is .04 c.c. make the new dilution as follows: To 8 parts of the 1 to 10 dilution add 2 parts of 0.9 per cent. saline. This gives us two hemolytic units in 0.1 c.c. of the new dilution.

Fourth, the remainder of the preliminary tests are made with the new two unit dilutions of each guinea-pig's serum as shown in the above table.

Place these tests three to seven in water-bath at 37° C. for one hour. Then add 0.2 c.c. of sensitized cells to each tube, and replace the tests in the water-bath for thirty minutes. Then read the reaction in each tube.

The reactions in preliminary tests are interpreted as follows:

### *Interpretation of the Preliminary Tests.*

Test No. 1. No trace of hemolysis indicates the absence of antisheep amboceptor, and the complement is O.K. in this respect. Any degree of hemolysis indicates the presence of natural antisheep amboceptor. Such a complement cannot be used for testing a new amboceptor, and for other tests only when the controls show that the amount of natural antisheep amboceptor present does not interfere with the fixation reaction. The natural antisheep amboceptor can be removed by absorption.[1]

Test No. 2. The 0.1 c.c. tube should show complete hemolysis within eight minutes, and the unit of hemolysis at the end of thirty minutes should be within the range of 0.06 to 0.03. If the hemolytic unit is 0.02 or 0.01 the complement is too active. This is a very rare occurrence. If the unit is 0.08, 0.09 or 0.1 it would be better to make a lower dilution in order to avoid pipetting the larger volume.

Test No. 3. Must be completely hemolyzed in 0.1, 0.2, 0.3 and have only a trace of fixation in 0.4.

Test No. 4. Must be completely hemolyzed.

Test No. 5. Must be completely fixed. If in ten pigs tested only one or two show a trace of hemolysis in the No. 5 test, they can be included in the pool, but any pig's serum giving only weak or no fixation should be excluded from the pool.

Test No. 6. Must be completely hemolyzed in eight to ten minutes.

Test No. 7. Must show no trace of hemolysis.

**To Find the Exact Dose of Pooled Complement for Diagnostic Tests.**—All complements found by the preliminary tests to be suitable for gonococcus fixation are pooled. This pooled product is stored, undiluted, in a sterile, neutral bottle, tightly stoppered. It will retain its hemolytic activity and fixability for three days, and sometimes longer, when kept in the ice-box at 0° to 4° C. It can be preserved for a much longer time by salting.

For diagnostic tests we use two hemolytic units of the gonococcus complement. By hemolytic unit of complement is meant the smallest volume of complement that at the end of thirty minutes in the water-bath at 37° C. will completely hemolyze 0.1 c.c. of sheep's erythrocytes that have been sensitized with three standard units of antisheep amboceptor. The pooled complement must be titrated every day with fresh sensitized cells. In this method for balancing the hemolytic system by a complement titration, instead of by a hemolysis titration, the hemolysin is used in its standard dilution every day. The new hemolysin is standardized by the method described (see attached note) and ever after is used in its standard dilution. The reason for this is that the titer of the hemolysin does not change, while every new complement is an unknown quantity both in regard to hemolytic activity and fixability until it has been tested. Therefore, in order that the various complements used for testing patients' serums from week to week may have as nearly as possible the same hemolytic and fixation value, we titrate the variable (the complement) against the constant (the hemolysin). As the new hemolysin was standardized with two hemolytic units of a known complement, the balance of the system is kept as nearly the same as possible for each day's tests.

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[1] To 1 c.c. of washed sheep cells add 1 c.c. of patient's serum. Place in ice-box for one hour. Then, centrifugalize and remove serum from cells. This serum will be free from natural antisheep amboceptor.

STANDARDIZATION OF THE HEMOLYTIC SYSTEM BY COMPLEMENT TITRATION.

| Tube No. | Complement. 1 to 10 dilution, c.c. | Saline, c.c. | Sensitized cells, c.c. | Five per cent. suspension. |
|---|---|---|---|---|
| 1 | .1 | .2 | .2 | 0 |
| 2 | .09 | .21 | .2 | 0 |
| 3 | .08 | .22 | .2 | 0 |
| 4 | .07 | .23 | .2 | 0 |
| 5 | .06 | .24 | .2 | 0 |
| 6 | .05 | .25 | .2 | 0 |
| 7 | .04 | .26 | .2 | 0 |
| 8 | .03 | .27 | .2 | 0 |
| 9 | .02 | .28 | .2 | 0 |
| 10 | .01 | .29 | .2 | 0 |
| 11 | 0 | .3 | .2 | 0 |
| 12 | .2 | .2 | 0 | .1 |

Dilute the complement 1 to 10 and titrate that dilution with 0.2 c.c. of sensitized cells. (This dose of sensitized cells contains 0.1 c.c. of 5 per cent. suspension of erythrocytes and three hemolytic units of antisheep amboceptor and saline solution to bring the total volume to 0.5 c.c. Place this titration in the water-bath at 37° C. and at the end of thirty minutes read the unit of hemolysis.

**The Antigen.**—All bacterial antigens are standardized by the method described on page 271, and the dose for diagnostic tests is always 0.1 c.c. of the standard dilution. It has been our experience that the antigenicity of our products has not diminished by standing. For example, our gonococcus antigens retain their original titer as long as they last. Some of them have been used for more than a year. A drop in the antigen unit indicates a weakly fixable guinea-pig serum and not deterioration of the antigen. The dilution for tests is placed in the water-bath at 56° C. for thirty minutes before using. This dilution can be used day after day if it is kept sterile and stored in the ice-box.

*The Test.*—The gonococcus test is made by the method described for the Wassermann test (p. 252). The tests are fixed for one hour in the water-bath at 37° C. To each tube is then added the dose of sensitized cells and the tests are returned to the water-bath for the hemolytic reaction in the control tubes. The fixation reactions are read when the system, antigen, and serum anticomplementary controls are completely hemolyzed. If the serum controls do not hemolyze within one hour, the test is not read. The tables on page 253 give the reactions in each tube of the test and all controls that indicate a true positive (four plus) or negative reaction and false positive or negative reaction.

**The Complement-fixation Test for Tuberculosis.**—Complement fixation has been studied in its relation to tuberculosis from several points of view; from the standpoint of its bearing on clinical medicine to discover whether it was of value in the diagnosis, prognosis or in controlling the specific therapy of tuberculosis in man; from the standpoint of pure pathology to determine what light it might throw upon the essential nature of the disease and immunity thereto; and from the position of the veterinarian who is in search of reliable diagnostic methods. The chief investigation has centered around the diagnostic and prognostic possibilities of the reaction. In the interest of the individual patient, as well as of public health, early diagnosis is essential, and the complement-fixation test has been sought as an aid in this direction. The earlier workers[1] on this subject reported such contradictory results that little hope was promised of practicable employment of the method. The more recent work, however, indicates an agreement of opinion upon the following points: The complement-fixation test gives a positive reaction in tuberculous infections only. The non-specific fixations with syphilitic serums reported by some investigators were probably due to imperfect antigens or to a coincident infection with tuberculosis.

The von Pirquet and complement-fixation tests do not run parallel.

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[1] The finding of complement fixation in tuberculous patients appears to be dependent upon the interplay of two, and possibly three, fundamental factors: (1) The antigens liberated in the body as a result of the invasion of the tuberculosis bacillus. The exact nature of these is still unknown. They may conceivably consist of the bacillus itself, of products of the disintegration of the bacillus, of endotoxins, or of products of the disintegration of the tissues evolved during the tuberculous process. (2) The antibodies which may be produced in response to the stimulus exerted by the antigens. (3) We must consider the role possibly played by the inhibiting substances that are stated by Calmette, and Caulfield, to occur in certain tuberculous serums, and which have the effect of producing negative reactions in serums which contain antibodies. The interaction of these factors in various quantitative relations may give rise to various hypothetical contingencies in the test reactions of a serum, the most important of which appear to be the following:

CASE 1.—No antigen is or has been present in the body, therefore we obtain a negative reaction since there is also no antibody.

CASE 2.—Antigen is present but for some reason there is no antibody formation, and the reaction is negative.

CASE 3.—Antigen is present, but the antibody is quantitatively insufficient to more than saturate the antigen; it therefore remains undetected, and the reaction is negative.

CASE 4.—Antigen and antibody are present, the latter in such excess that it can be detected, and a positive reaction results.

CASE 5.—Both are present, but there is also an inhibiting substance which interferes with the reaction of fixation, and therefore the result is negative.

CASE 6.—The antigen formerly present has disappeared and no more is being liberated, but the antibody evoked by it still remains, being demonstrable in decreasing amounts on successive examinations.

Considered in the light of these hypothetical possibilities, the observed discrepancies in the result in examining a series of patients become more reasonable and susceptible of interpretation.

Many of the incipient or normal cases having a positive tuberculin reaction give no fixation of complement. If a normal case having a positive von Pirquet gives a positive complement-fixation reaction this should not be considered to be a "false positive," but the case should be kept under observation for the appearance of clinical symptoms.

With a suitable antigen a positive complement-fixation reaction will be found in from 75 per cent. to 95 per cent. of clinically active pulmonary cases.

A negative complement-fixation reaction in any stage of the disease does not invalidate the test, as it may have great significance for prognosis and treatment.

Studies now in progress in this laboratory corroborate the above conclusions in regard to specificity, the discrepancy between von Pirquet and complement-fixation reactions and the incidence of positive reactions in clinically positive cases. Further than that, we have found that the antigens made from the whole bacillus, either moist suspensions or defatted dried bacillus suspensions, are superior to any of the extracts we have tried. We had no fixation with alcoholic extracts.

The observation of von Wedel[1] that a clinically positive case giving a negative reaction twenty-four hours after bleeding may give a positive reaction on the seventh day after bleeding has been corroborated by us in a study of 500 cases; but we have not been able to prove that this has any diagnostic significance. Further study will have to determine the specific value of this "later" reaction.

Recent studies carried out at Trudeau Sanatorium have called attention to the value of the complement-fixation test in the management of the life of a tuberculous patient. A patient having a negative complement-fixation test can be allowed to exercise much more freely than a patient who has a positive complement fixation.

McIntosh and Fildes,[2] using an emulsion of fresh, living tubercle bacilli, found 80.7 per cent. complement-fixation reactions in orthopedic cases and 37.5 per cent. positive in glands.

In this laboratory, using the defatted antigen described above, we have found 22 per cent. positive in bone and joint diseases and 58 per cent positive in glands. Keller and Moravek[3] state that they can differentiate between human and bovine infection in orthopedic cases, but we have not had that experience.

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[1] Jour. Im., No. 5, iii.

[2] Lancet, 1914, lcxxxv, 485.

[3] Int. Jour. Surg. July, 1915.

Other references are:

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Calmette and Massol: Compt. rend. Soc. de biol., 1912, lxxv, 120.
Burns, W. B.: Jour. Am. Med. Assn., lxviii, 1386.
Corper: Jour. Infec. Dis., xix, 315.

Bang and Anderson report a series of tests with tuberculous and non-tuberculous cattle. They state that strong reactions have diagnostic significance.

Very little work has been reported upon complement-fixation tests on cattle, and those reports indicate that the methods employed were not perfectly balanced.

**Technic of the Test.**—The method for making the tuberculosis complement-fixation test is the same as that described for the gonococcus test. The fixation is carried out in the water-bath for one hour at 37° C.

**The Complement-fixation Test for Glanders.**—Complement fixation has proved to be a valuable aid in the diagnosis of glanders. It is generally considered specific and more reliable than the agglutination test. In this laboratory the antigen is prepared from a twenty-four-hour growth of B. mallei on salt-free veal-agar 1.6 per cent. acid. The growth is washed off with sterile distilled water, autolyzed for six to eight hours at 80° C., filtered through paper pulp and a Berkefeld, and the filtrate sterilized at 56° C. on three successive days for a half-hour. This antigen, like the gonococcus and all other aqueous extracts, must be made isotonic before use. The test is performed like the others, but the optimum period for fixation has been found to be six to eighteen hours in the ice-box. The New York Health Department condemns all horses that give a + + + + complement-fixation reaction when it is confirmed by the eye mallein test, while those that give a strongly + + and + + + are suspected of having a slight glanders infection.[1]

**Streptococcus Infections.**—The value of complement fixation in the diagnosis of streptococcus infections is still uncertain. In this laboratory fairly satisfactory results are obtained with a saline antigen prepared as follows: A twenty-four-hour growth on salt veal-agar neutral to phenolphthalein is washed off with 0.85 per cent. saline solution. The emulsion is heated at 60° C., one hour, left in the ice-box twenty-four hours, and centrifugalized. The supernatant fluid is used for tests, either water-bath or incubator being used for fixation. The test seems to be specific, but is far from perfected.

**Pertussis.**—Complement fixation has been used for the determination of the etiological cause of whooping-cough and for the diagnosis of the disease. The results of many investigators, including ourselves, confirm the findings of Bordet, that the Bordet-Gengou bacillus is the etiological cause of whooping-cough. As to the diagnostic value of the test, reports vary. The figures given by the workers who use active serum are undoubtedly too high, as investigations in this laboratory have proved that active serum may give non-specific fixation with even a highly specific antigen. We have found the most satisfactory antigen, that is, the strongest and most specific to be obtained by shaking and then autolyzing at 56° C. A twenty-four-hour culture on the Bordet-

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[1] At autopsy, macroscopic lesions are always shown by horses giving a + + + + complement-fixation test, rarely by those giving a + + or a + + + reaction.

Gengou potato-blood-agar medium is scraped off with a platinum spud and put in sterile distilled water. The emulsion is shaken for about two hours, left in a thermostat at 56° C. for about eighteen hours, and centrifugalized. The supernatant fluid is used for tests, being made isotonic immediately before using. Water-bath or incubator is used for fixation; room temperature, at which some workers allow fixation to take place, give such variable results as to be unsuitable for routine work. We obtain a positive reaction in about 50 per cent. of whooping-cough cases in the paroxysmal stage. The administration of whooping-cough vaccine may increase the strength of the complement-fixation reaction, but experiments with normal individuals have proved that vaccine in itself, in the absence of whooping-cough, does not bring about a positive reaction. Immune serum of high antibody content may be produced by the intraperitoneal inoculation of rabbits once a week with a live culture of the Bordet-Gengou bacillus, beginning with a dose depending on the condition of the rabbit. The height of immunity is usually reached through five or six inoculations; if other inoculations are given, the antibody content decreases The rabbits are bled nine days after the last inoculation.

**Meningitis.**—The complement-fixation method has been successfully applied in epidemic meningitis by Brück, but the diagnosis can more readily be made by the bacteriological examination of cerebrospinal fluid. Complement-fixation is a valuable means of differentiating strains of meningococci. The antigen used here for this purpose is prepared in the same manner as gonococcus antigen. Water-bath fixation is used for all titrations.

**Typhoid.**—Complement fixation is a valuable method for corroborating the Widal. Its exact clinical value and specificity have not yet been established. The use of a highly polyvalent antigen is essential. Garbat's method of preparing the antigen is to wash off with sterile distilled water a twenty-four-hour growth on agar. Heat the emulsion at 60° to 70° C. for twenty-four hours, shake with glass beads for twenty-four hours, and centrifugalize until the supernatant fluid is absolutely clear. A positive reaction usually appears only after bacteria have disappeared from the circulation; it becomes stronger during convalescence and persists for several months afterward.

**Parasitic Skin Diseases.**—Kolmer and Strickler,[1] corroborated by others, reported rather favorable results of fixation of complement in ringworm and favus.

## APPENDIX.

### PREPARATION AND STANDARDIZATION OF ANTIGEN AND OF IMMUNE SERUM.

**Antigen.**—The method of preparing antigen depends on the nature and on the purpose of the test to be made. It may be said in general that antigens are of two types: (1) specific bacterial or other cellular antigens, or protein extracts, and (2) non-specific lipoidal antigens (used in the Wassermann reaction). The methods of preparing these two types of antigens also differ. Each method is given in detail below.

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[1] Kolmer and Strickler: Complement Fixation in Parasitic Skin Diseases, Jour. Am. Med. Assn., 1915, lxiv, 800.

*Lipoidal Antigens.*—These antigens are used in the Wassermann reaction for syphilis (*Q. V.*)

*Crude Alcoholic Heart Antigen.*—In our laboratory a most satisfactory antigen is prepared as follows: hearts (from guinea-pigs bled to death for complement) are minced and washed in tap water until free from blood, and macerated in 96 per cent. ethyl alcohol in the proportion of 1 gram of heart to 5 c.c. alcohol. Extraction is allowed to take place in the ice-box for at least three months, and then in the incubator for a week. The extract is filtered through paper and kept in the ice-box, where it remains stable for at least a year.

The antigen should be diluted and standardized according to the scheme given below. (See Antigen Titration.) In diluting, at least 1 c.c. of the extract should be placed in a bottle or flask, and the first 10 c.c. of salt solution are added, drop by drop, the remainder slowly, the mixture being effected by gently rotating the receptacle, not by shaking. If these directions are observed carefully, a turbid suspension will result. A lipoidal antigen which does not yield a turbid dilution will not fix complement satisfactorily, and should be discarded. The titer of lipoidal antigens prepared as above varies from 1 to 20 to 1 to 100.

Noguchi's acetone insoluble fraction of beef heart, liver, or kidney, may be prepared by extracting macerated tissue with ten times the amount of absolute alcohol at room temperature for several days, filtering, evaporating the filtrate to dryness, taking up the residue with ether, treating the ethereal solution with five times its volume of acetone, and making a saturated solution of the precipitate in absolute methyl alcohol.

Cholesterinized antigen may be prepared from the crude alcoholic heart antigen described above. This crude alcoholic antigen is divided into two parts. The first part is set aside. The second part is heated in a water-bath to 40° C. and cholesterol crystals are added to saturation (about 0.8 per cent.). It is now placed in the incubator at 37° C. for twenty-four hours, to insure saturation, and then cooled to room temperature. This will precipitate out excess cholesterol, which is removed by filtration. The saturated cholesterinized antigen is now combined in equal volumes with the first part of the crude alcoholic antigen. Half saturation with cholesterol results, and the mixture constitutes the finished cholesterinized antigen. It should be standardized and appropriately diluted for use. Such antigens frequently show a tendency to be oversensitive in binding complement, especially in ice-box fixation. For this reason, in performing actual tests where they are used, the method of fixation should be that of one hour at 37.5° C. (incubator).

*Preparation of Tuberculosis Antigen.* — *Defatted dried-bacillus antigen.*—The bacilli are grown in glycerin-broth[1] for three months. The culture is then killed by heating in the Arnold sterilizer for one hour. The culture is filtered through filter paper. The filtrate is discarded, and the residue treated as fol-

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[1] *Formula for Tuberculin Broth.*

| | |
|---|---|
| Lean beef (chopped) . . . . . . . . . . . . . . | 20 pounds |
| Water, tap . . . . . . . . . . . . . . . . . | 20 liters |
| Soak overnight in ice-box. Next morning strain through cheese-cloth. | Add: |
| Witte's peptone (1 per cent.) . . . . . . . . . . . | 200 grams |
| Sodium chloride (0.5 per cent.) . . . . . . . . . . . . | 100 grams |

Heat to 50° C. to dissolve the peptone, rubbing it with hands in broth. Titrate and add normal sodium hydroxide to set reaction about 1.3 acid to phenolphthalein. Boil for three-quarters of an hour and make up with water to 20 liters. Titrate again (as above) and adjust reaction if necessary to 1.4 acid to phenolphthalein. Filter through paper and add 5 per cent. glycerin. Put 225 c.c. in each quart Blake bottle. Autoclave at 15 pounds pressure for three-quarters of an hour. Finish reaction to 1.4 to 1.6 acid.

Hot titration (end-reaction being pink color, which persists on boiling).

lows: It is first washed in sterile water to remove all traces of broth. Then it is placed in absolute alcohol in the proportion of one volume of residue to ten volumes of alcohol. This mixture is placed in the ice-box for two weeks. It is then centrifugalized for ten minutes and the supernatant fluid discarded. To the sediment is added ten volumes of fresh absolute alcohol. This mixture is placed in the water-bath at 56° C. for one hour.[1] It is then centrifugalized for ten minutes, and the supernatant fluid is discarded. To the residue add ten volumes of ether. This mixture is stirred constantly at room temperature for one hour. Centrifugalized ten minutes, and the supernatant fluid is discarded. The centrifuge tube containing the residue is plugged with sterile cotton and placed in the dark at room temperature for twenty-four to forty-eight hours, until the residue is perfectly dry. This is usually accomplished in twenty-four hours. The dried powder is emulsified in a large mortar by trituration with 0.9 per cent. saline solution, in the proportion of 1 gram of powder to 200 c.c. of saline. The emulsion is heated for one hour at 80° C. The antigen is now ready for use, and is standardized as described below in this section.

*Moist-Bacillus Antigen.*—The bacilli are grown as described above. The broth culture is placed in the Arnold sterilizer for one hour. It is then filtered through folded paper and the filtrate is discarded. The residue is washed in sterile 0.9 per cent. saline solution to remove all traces of the broth. It is then centrifugalized for ten minutes and the supernatant salt discarded. The moist residue is emulsified by trituration with saline in a large mortor. This emulsion is heated for one hour in water-bath at 80° C. It is now ready for use, and is standardized by the method described above.

*Preparation of Other Bacterial Antigens.*—In this laboratory all bacterial antigens are made by the method described for the tuberculosis residue, the only variation for the different organisms being the culture medium and the period of growth.

*Gonococcus.*—A polyvalent antigen is made from the ten strains isolated by Torrey.

*Culture Method.*—The gonococci are grown on potato-tube slants of glycerin-veal-horse-serum agar for forty-eight hours. Then plates of the same medium are inoculated with the whole growth of the slant culture. These plates are incubated for forty-eight hours. The cultures are examined by smear for purity of growth. The growth is scraped to the center of the plate with a sterile glass slide and this mass is transferred by means of a platinum loop to a centrifuge tube containing 15 c.c. of absolute alcohol. The growth on all plates is placed in one centrifuge tube. This mixture is placed in the water-bath at 56° C. for thirty minutes, stirring constantly with a glass rod. It is then treated in the same manner as described for tuberculosis antigen residue. (See Defatted dried-bacillus antigen.)

*Meningococcus.*—An antigen as nearly polyvalent as possible is made from twelve or more strains.

*Culture Method.*—The meningococci are grown by the method described for gonococci, except the period of growth. The meningococci are grown for twenty-four hours.

*Glanders.*—An antigen from a number of strains is made.

*Culture Method.*—The bacilli are grown on glycerin-potato agar for twenty-four hours. The method for making the antigen is the same as for the gonococcus.

*Pertussis.*—Culture method same as for gonococcus.

*Influenza.*—A mixed antigen is made from twelve or more strains.

*Culture Method.*—The influenza bacilli are grown on coagulated horse-serum agar for forty-eight hours. The antigen plates are made from same medium. The growth on plates is treated by the method described for gonococcus antigen.

*Typhoid.*—Prepared in same manner as meningococcus antigen.

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[1] If the supernatant alcohol has any trace of color, the alcoholic washings are repeated until the alcohol is colorless.

*Standardization of Antigen.*—There are two requirements for a good antigen, (1) a long range and (2) specificity.[1] The range of an antigen is determined by mixing varying amounts of the antigen with a constant amount of a previously tested homologous immune serum, a constant amount of complement and a constant amount of sensitized erythrocyte suspension. The specificity of an antigen is determined by using heterologous instead of homologous immune serum in the titration. The methods employed in our laboratory in the titration of antigen are given below. Readings are made after the erythrocytes have settled, and meantime the titration should be kept in the ice-box to prevent a continuation of hemolysis. An immediate reading may be made by centrifugalizing the tubes. If fixation is complete through 0.025 c.c. (tube 6), a higher dilution of the antigen should be made, and titrated in the same manner to determine the unit of antigen. This is the smallest amount of antigen that, with two units of homologous immune serum (or 0.01 c.c. of a human serum which has given a + + + + reaction, according to our scheme) gives complete fixation of complement. The anticomplementary dose is the smallest amount of antigen that is in itself inhibitory of hemolysis. The longer the range of the antigen the greater is the probability of success in diagnosis. An amount of antigen that fixes complement completely with a serum of high antibody content may give incomplete or no fixation with a serum of low antibody content. Hence in making diagnostic tests, where the detection of even a small amount of antibody is desired, it is advisable to use much more than one unit of antigen. As the maximum amount of antigen that may be used with safety is one-fourth the anticomplementary dose, an antigen of long range is necessary. If in the tables below inhibition is complete (Plate V, *a* and *b*) in the first five or six tubes, and if hemolysis is complete (Plate V, *e*) in tubes 8 and 12, and there is very slight inhibition (Plate V, *d*) in tube 7 only, then 0.1 c.c. is the amount of antigen to be used in diagnostic tests. It is usually convenient to dilute the antigen so that 0.1 c.c. is the amount used. If one-fourth the anticomplementary dose gives complete fixation with a heterologous immune serum the antigen is non-specific and unsuitable for tests. Occasionally an antigen is lytic for erythrocytes. In such a case tubes containing the largest amount of antigen show more hemolysis than those containing less. The fixation curve instead of dropping (Fig. 93, *a*) first rises and then drops again (Fig. 93, *b*). If a lytic antigen is also anticomplementary and has a long fixation range it may be used, otherwise it should be discarded.

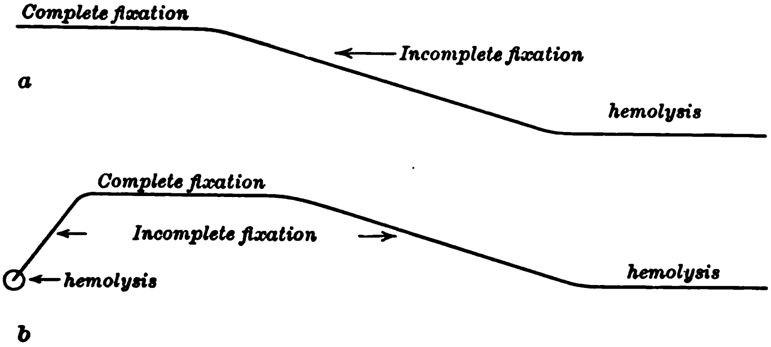

FIG. 93

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[1] The range of an antigen is the difference between the anticomplementary dose (the smallest amount of antigen that is in itself inhibitory) and the minimum fixing dose, the antigen unit.

ANTIGEN TITRATION (FOR ALL MICROBAL ANTIGENS).

| Number of tube. | Immune serum, c.c. | Antigen, c.c. | Ten per cent. complement, c.c. | 0.85 per cent. saline, c.c. | | Sensitized erythrocyte suspension, c.c. | |
|---|---|---|---|---|---|---|---|
| 1 | 0.01 | 0.25 | 0.1 | 0.0 | Incubated one-half hour in the water-bath at 37° C. | 0.2 | Incubated one hour in the water-bath at 37° C. |
| 2 | 0.01 | 0.2 | 0.1 | 0.0 | | 0.2 | |
| 3 | 0.01 | 0.15 | 0.1 | 0.05 | | 0.2 | |
| 4 | 0.01 | 0.1 | 0.1 | 0.1 | | 0.2 | |
| 5 | 0.01 | 0.05 | 0.1 | 0.15 | | 0.2 | |
| 6 | 0.01 | 0.025 | 0.1 | 0.2 | | 0.2 | |
| 7 | 0.0 | 0.4 | 0.1 | 0.0 | | 0.2 | |
| 8 | 0.0 | 0.3 | 0.1 | 0.0 | | 0.2 | |
| 9 | 0.0 | 0.2 | 0.1 | 0.05 | | 0.2 | |
| 10 | 0.0 | 0.1 | 0.1 | 0.1 | | 0.2 | |
| 11 | 0.0 | 0.05 | 0.1 | 0.15 | | 0.2 | |
| 12 | 0.02 | 0.0 | 0.1 | 0.2 | | 0.2 | |

**Obtaining of Immune Serum.**—The method of withdrawing immune serum from an animal depends on the animal and the purpose of bleeding. In the case of a horse the bleeding is made from the jugular vein. Rabbits are bled from the marginal ear vein if only a few cubic centimeters (less than 5 c.c.) of serum are required. If a larger amount is necessary the rabbit is etherized, tied on a board made for that purpose and under aseptic precautions bled to death from the carotid artery. A cannula may be inserted in the artery and the blood allowed to flow into one or more tubes. To obtain the maximum amount of serum the tube of blood should be slanted at room temperature until coagulation has taken place.

In obtaining blood for complement-fixation tests on human beings venous puncture is the most satisfactory method. The drawing of sufficient blood from finger-tip or ear-lobe is a tedious process and the blood cells are liable to be somewhat broken, so that the serum is tinged with hemoglobin and rendered unfit for testing.

The specimen for test is most easily obtained from the median basilic vein of the elbow. The arm should be rendered aseptic by the usual method of scrubbing with soap and water and the application of a 1 to 2000 bichloride of mercury pack for ten minutes. The site of puncture should then be rubbed with alcohol and ether a ligature is placed above the elbow sufficiently tight to fill the vein, but not tight enough to impede the arterial circulation. A sterile needle is then introduced and 5 to 10 c.c. of blood allowed to flow into a sterile test-tube, which is corked and left slanted at room temperature until the blood is firmly clotted. The tube should then be placed in the ice-box until the serum has separated. If it is necessary to mail a blood specimen, serum only should be sent, as lysis of the blood cells would be caused by the heat and shaking to which the specimen would be exposed in transit. The serum should be mailed in a sealed ampoule or tightly corked tube.

Immune serum may be separated from erythrocytes and fibrin by centrifugalization before coagulation has taken place; or, better, by pipetting or pouring from the clot after coagulation. Serum obtained by the second method may be entirely freed from erythrocytes by centrifugalization. It should be removed from the clot before spontaneous lysis of the red blood cells occurs, as hemoglobin has the power of fixing complement in itself; a hemolyzed specimen of serum (one containing hemoglobin) is therefore anticomplementary, *i. e.*, it inhibits hemolysis without the presence of a specific antigen, and hence cannot be tested for a specific amboceptor. Serum, both before and after removal from the clot, should be kept in a cool place, at a temperature not higher than 0° C., as the antibody content is lost more rapidly at a high than at a low temperature. For practical purposes serum is best preserved by freezing.[1] The addition of a preservative is not advisable, as the accuracy of the test may thereby be invalidated. Contamination of an immune serum should be avoided, as it may result in an anticomplementtary and non-specific action of the serum.

All immune serum before use in tests should be inactivated, *i. e.*, heated for one-half hour at 56° C., to destroy complement and serum components that may give rise to non-specific fixation. Spinal fluid, however, should be used without being inactivated, as the amount of complement in it is negligible.

**Standardization of an Immune Serum.**—An immune serum may be standardized by means of an antibody content titration. This is made for the purpose of measuring as accurately as possible the amboceptor content of the serum. Our technic is given in the table below. The serum is first titrated in a 10 per cent. dilution; if fixation is complete through 0.01 c.c. (tube 10) a similar titration of a 1 per cent. dilution is made. The amount of antigen used is double the antigen unit; for example, if 0.05 c.c. of a 10 per cent. dilution of antigen is the smallest amount giving complete fixation with a homologous immune serum, 0.1 c.c. of that dilution is used in the antibody content titration. The reading is made as in an antigen titration (*q. v.*) after the erythrocytes have settled. The antigen and serum controls (tubes 11 and 12) should, of course, be hemolyzed. An *antibody unit* is the smallest amount of serum that with the two units of a homologous antigen gives complete fixation of complement. The number of antibody units per cubic centimeter may be calculated by dividing 1 c.c. by the minimum fixing dose; that is, if 0.05 c.c. of a 1 in 10 dilution is the antibody unit, the number of antibody units per cubic centimeter is $\frac{1}{0.005} = 200$. An antibody content titration is used for the standardization of antimicrobic sera, such as antigonococcus and antimeningococcus horse sera.

The tests upon which the standardization of a new serum is based consist of titrations of the new bleeding in comparison with the standard titer of a known serum. In order that the comparison may be true, it is necessary that the reagents used in making the tests shall be as nearly as possible constant in their reactions, leaving as the only variable the unknown serum to be tested. This would be a simple matter were it not for the fact that the complement is also a variable factor, and attempts to render it constant are not always successful. It is true, then, in this as in all other complement-fixation work, that the value of the tests depends upon the accurate measurement of the dose of complement. Formerly[1] [2] this complement dose was measured in regard to its hemolytic strength, an exact hemolytic unit being used with two units of hemolysin. If the fixation and hemolytic units of a given complement were always proportionate this method for determining the complement dose would be an advantage, as a clear-cut hemolytic reaction is quickly and easily obtained when the complement is in good condition. On the other hand[3] the hemolytic and fixation strengths of guinea-pig serums are many times diverse, and to measure the dose of complement by its hemolytic strength will not give an accurate fixation titer of the new immune serum. The method described below is now employed in this laboratory for the standardization of our meningococcus horse serums.

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[1] Serum is perfectly preserved by evaporating to dryness in a vacuum desiccator, but the procedure is complicated.

ANTIBODY CONTENT TITRATION.

| Number of tube. | Immune serum diluted 1 to 10 (etc.), c.c. | Antigen in standard dilution, c.c. | Complement in dilution as determined by titration, c.c. | 0.85 per cent. saline, c.c. | | Sensitized erythrocyte suspension, c.c. | |
|---|---|---|---|---|---|---|---|
| 1 | 0.1 | 0.1 | 0.1 | 0.0 | Incubated one hour in the water-bath at 37° C. | 0.2 | Incubated one hour in the water-bath at 37° C. |
| 2 | 0.09 | 0.1 | 0.1 | 0.01 | | 0.2 | |
| 3 | 0.08 | 0.1 | 0.1 | 0.02 | | 0.2 | |
| 4 | 0.07 | 0.1 | 0.1 | 0.03 | | 0.2 | |
| 5 | 0.06 | 0.1 | 0.1 | 0.04 | | 0.2 | |
| 6 | 0.05 | 0.1 | 0.1 | 0.05 | | 0.2 | |
| 7 | 0.04 | 0.1 | 0.1 | 0.06 | | 0.2 | |
| 8 | 0.03 | 0.1 | 0.1 | 0.07 | | 0.2 | |
| 9 | 0.02 | 0.1 | 0.1 | 0.08 | | 0.2 | |
| 10 | 0.01 | 0.1 | 0.1 | 0.09 | | 0.2 | |
| 11 | 0.0 | 0.2 | 0.1 | 0.0 | | 0.2 | |
| 12 | 0.2 | 0.0 | 0.1 | 0.0 | | 0.2 | |

*Technic.*—A mixed or, when possible, a polyvalent, antigen made from the strains from which the immune serum was produced is used. This antigen is made by the method described on page 261 for a defatted, dried coccus antigen. The antigen is always used in its standard dilution.

The complement from each guinea-pig is given the preliminary tests described on page 265. After the results of the preliminary tests are obtained, those complements which are found to have adequate fixation and hemolytic strength for meningococcus fixation are pooled and the pooled complement is tested to determine the exact dose to be used. This is accomplished by titrating the standard dilution of a known meningococcus serum with two standard units of meningococcus antigen and with varying dilutions of the pooled complement. For the standard dose of complement to be used in testing the unknown serums we use

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[1] Olmstead and Luttinger: Complement Fixation in Pertussis, Arch. Int. Med., 1915, xvi, 67.

[2] Hitchens: [3] Wilson: Proc. New York Path. Soc., N. S., Nos. 6–8, vol. xvii.

0.1 c.c. of that dilution of the pooled complement which gives the standard titer of the known serum in the required time and complete hemolysis of all hemolytic controls within thirty minutes.

**Titration of Unknown Serums.**—Having found the most favorable dose of complement the unknown serums are titrated with that in the presence of two standard units of the meningococcus antigen. (Page 274 for serum titration method.) The horse serums are stored without preservative. They are inactivated by heating in the water-bath at 56° C. for thirty minutes. Each bleeding is titrated in 1 to 10 and 1 to 100 dilution. Up to the present time the titers of our horses have been found within that range.

The titrations are placed in the water-bath at 37° C. for thirty minutes for fixation of the complement. At the end of that time the indicator, consisting of 0.1 c.c. of sheep cells sensitized with two units of hemolysis, is added and the tests are replaced in the water-bath for one hour. The reactions are read at the end of one hour by the reading method employed for diagnostic tests. If desired the titrations may be placed in the ice-box overnight and the reactions read from the supernatant fluid in the morning.

# PART II.

# PATHOGENIC MICROÖRGANISMS INDIVIDUALLY CONSIDERED.

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## CHAPTER XV.

## THE PATHOGENIC MOULDS (HYPHOMYCETES, EUMYCETES) AND YEASTS (BLASTOMYCETES).

### THE HYPHOMYCETES.

THE majority of the moulds are not pathogenic for human beings, and interest us more as organisms which are apt to infect foodstuffs and media. Some are, however, true parasites, and produce a number of rather common diseases; for example, ringworm, favus, thrush, and pityriasis versicolor. Certain of the commoner moulds (mucor, aspergillus) have also been reported from time to time as present in pathogenic conditions in man as well as in the lower animals. Many varieties have been found in plant diseases, and some may be a source of danger to man indirectly. Indeed, when they form poisonous substances, as in the infection of grain by *Claviceps purpurea* (ergot poisoning), they are distinctly dangerous.

Paltauf reported the case of a man who died after enteritis with secondary peritonitis. The autopsy showed multiple abscesses in brain and lungs, besides the lesions in the intestines and peritoneum, in all of which a species of mucor was found. Two other cases of primary mucor infection in humans were reported by Furbringer. A number of species of mucor have been found in ear and eye infections; for example, Mucor corymbifer (Fig. 94) has been found in ophthalmia. A number of species of moulds are pathogenic for lower animals. Aspergillus is found thus very frequently in lower animals, especially in birds, where a kind of pseudotuberculosis is often produced. Quite a number of similar cases have been reported in man, and it is supposed that the infection may be carried from birds to man. Aspergillus nodulans (branched sterigmata) and aspergillus fumigatus (Fig. 95) are the most frequent varieties found. Penicillium minimum (similar to glaucum, Fig. 96) has been found by Liebermann in inflammation of the ear.

The more common pathogenic forms for man are those producing the various hair and skin lesions mentioned above. These moulds are all classed with the fungi imperfecti. The claim that "trench foot" was due to a mould[1] has not been substantiated.[2] The moulds described as causing "trench foot" have been shown to be only secondary invaders [1] (See p. 27 for general characteristics of moulds; see also Plate I.)

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[1] Raymond and Parisot: Presse méd., October 19, 1916, p. 464.

[2] Turner: Lancet, 1917, cxciii, 638.

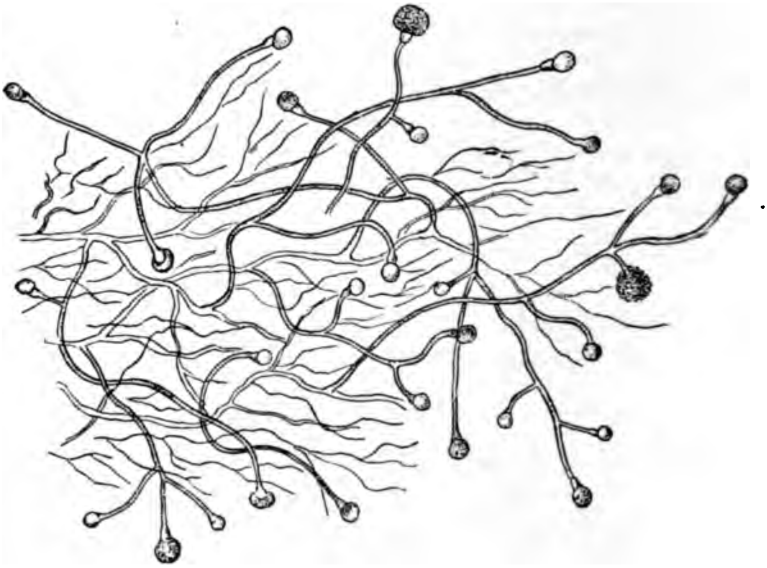

FIG. 94.—Mucor corymbifer, Coh. Mycelium with underlying branched carriers, × 270 diam. (After Lichtheim.)

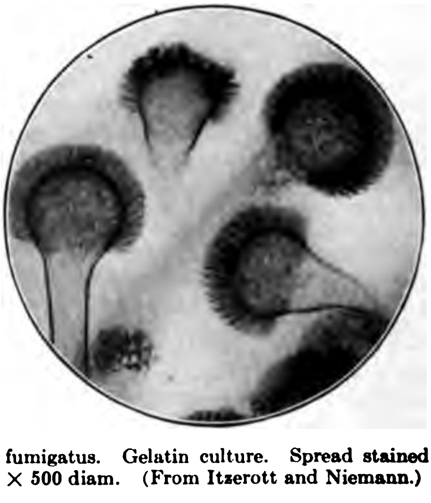

FIG. 95.—Aspergillus fumigatus. Gelatin culture. Spread stained with gentian violet. × 500 diam. (From Itzerott and Niemann.)

**Trichophyton (Tinea, Ringworm Fungus).**—Ringworm of the body or hairless parts of the skin (called also tinea circinata) and ringworm of the hairy parts (called also tinea tonsurans, herpes tonsurans or sycosis) are due to several species of the imperfecti group (p. 28).

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[1] Sweet, Norris and Wilmer: Lancet, 1918, i, 564. Wilson, B.: Internat. Am. Med. Museum Bull., 1918, No. 7, 202. Cobbett: War. Med., 1918, ii, 707.

According to Sabouraud,[1] whose conclusions are based on an extensive series of microscopic examinations of cases of ringworm in man and animals, of cultivation in artificial media, and of inoculation on man and animals, there are two distinct groups of the fungi causing ringworm in man—one with small spores (2μ to 3μ) which are known as *microspora*, and one with large spores (7μ to 8μ) which are called *megalospora*. They differ in their mode of growth on artificial media and in their pathological effects on the human skin and its appendages. The small-spored varieties or T. microsporon, are the common fungus of Tinea tonsurans of children, especially of those cases which are rebellious to treatment, and their special seat of growth is in the substance of the hair. T. megalosporon (Fig. 97) is essentially the fungus of tinea circinata, or ringworm of the beard and of the smooth part of the skin; the prognosis as regards treatment is good. One-third of the cases of T. tonsurans of children are due to megalospora. The spores of T. microsporon are contained in a mycelium; but this is not visible, the spores appearing irregularly piled up like zoöglea masses; and, growing outside, they form a dense sheath around the hair. The spores of T. megalosporon are always contained in distinct mycelium filaments, which may either be resistant when the hair is broken up or fragile and easily breaking up into spores. The two groups show distinct and constant characters when grown in artificial cultures. The cultures of T. microsporon show a downy surface and white color; those of T. megalosporon a powdery surface, with arborescent peripheral rays, and often a yellowish color. Although the morphological appearances, mode of growth, and clinical effects of each group of trichophyton show several similar characters in

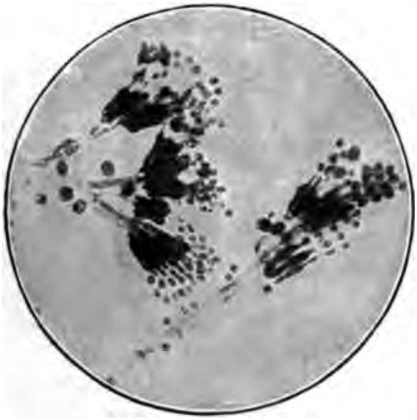

FIG. 96.—Penicillium glaucum. Gelatin culture. Spread stained with gentian violet. 500 : 1. (From Itzerott and Niemann.)

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[1] Ann. de Dermat. et de Syph., 1892 and 1893.

general yet there are certain constant minor differences in strains which point to the fact that there are a number of different varieties of trichophyton included under each group. The varieties included under T. microsporon are few in number and, with the exception of one which causes the common contagious "herpes" of the horse, almost entirely human. The varieties of T. megalosporon are numerous and fall into several natural subgroups, the members of which resemble one another

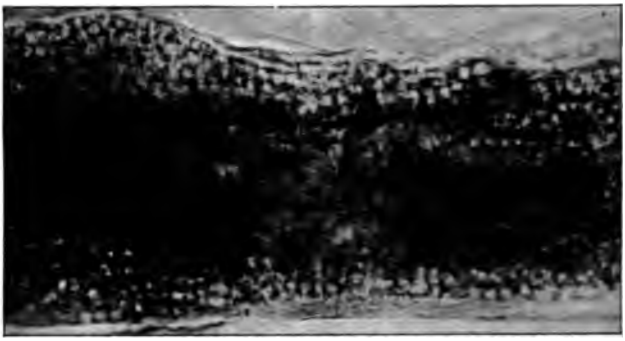

FIG. 97.—Hair riddled with ringworm fungus. Megalosporon variety.

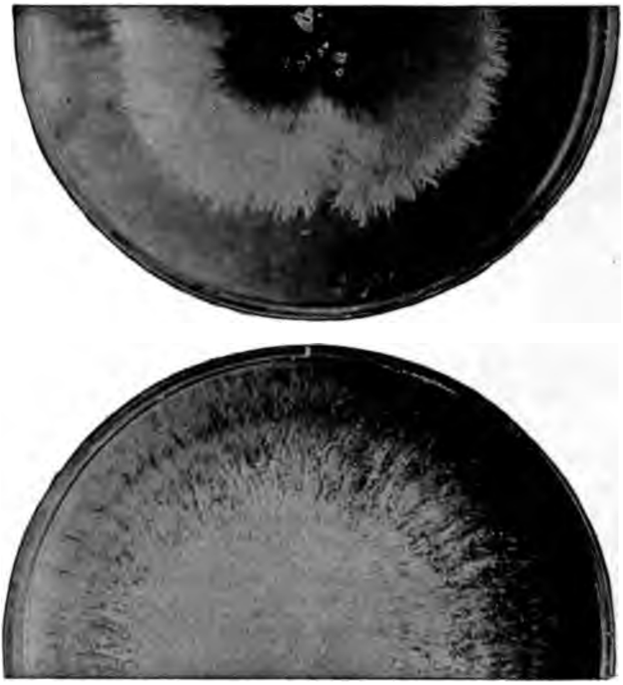

FIG. 98.—These two half-plates show three months' growth on peptone-maltose agar of two megalosporon varieties of the ringworm fungus. Natural size.

both from clinical and mycological aspects (Fig. 98). Cultures of any strain inoculated into guinea-pigs and other laboratory animals may produce infection.

A number of cases of eczema-like lesions of volar surfaces in man have been shown to be due to a species of ringworm fungus.[1]

**Achorion Schoenleinii (Favus).**—Favus is due to a fungus discovered by Schoenlein in 1839, and called by Remak Achorion schoenleinii. The disease is communicated by contagion, the fungus being often derived from animals, especially cats, mice, rabbits, and fowls; dogs also are subject to it. Buchanan[2] calls attention to the possibility of mouse favus being carried in Australian or other wheat that has been stored for any time and thus made liable to contamination by diseased mice.

The fungus grows much more slowly than the ringworm fungus, and is therefore not so quickly transmitted but it will surely infect careless, dirty people coming in daily contact with it. Want of cleanliness is a predisposing factor. The fungus seems to find a more favorable soil for its development on the skin of persons in weak health, more in those suffering from phthisis than from other diseases.

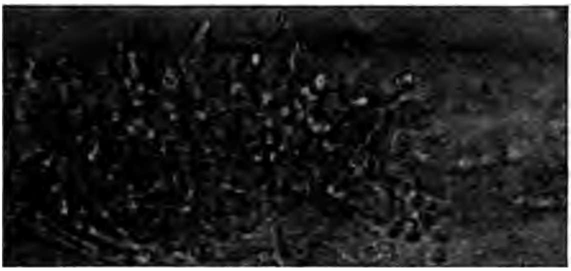

FIG. 99.—A portion of a favus-infected hair; magnified.

Favus produces tissue irritation of a very chronic course and of great resistance to treatment. The spores generally find their way into the hair follicles, where they grow in and about the hair (Fig. 99). It grows in the epidermis, the density of the growth causing pressure on the parts below, thus crushing out the vitality of the hair and giving rise to atrophic scarring. The disease shows a marked preference for the scalp, but no part of the skin is exempt, and even the mucous membranes are likely to be attacked. Kaposi has reported a case in which a patient suffering from universal favus died, with symptoms of severe gastro-intestinal irritation, which was found after death to be due to the presence of the favus fungus in the stomach and intestines. Foster[3] reported many cases of favus and ringworm of the nails in immigrants. On the scalp it first appears as a tiny sulphur-yellow disk or scutulum, depressed in the center like a cup and pierced by a hair. This is the characteristic lesion. The cup shape is attributed by Unna to the fact that growth is more vigorous at the sides than at the center. Under the microscope material from a scutulum teased out in a drop or two of 20 per cent. sodium hydrate solution (slightly heated) shows chiefly small doubly contoured, round or oval spores, single or in chains scattered throughout a dense network of fine threads among which on the edge of the scutulum may be recognized distinct branched hyphæ with swollen ends.

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[1] For method of examination of these fungi, see Microscopic Methods, p. 88.

[2] Ormsby and Mitchell: Jour. Am. Med. Assn., 1916, lxvii, 711. Jour. Am. Med. Assn., 1919, lxxii, 97.

[3] Jour. Am. Med. Assn., 1914, lxiii, 640.

The favus fungus is readily cultivated at the body temperature, less readily at room temperature, in the ordinary culture media, as agar, blood serum, gelatin, bouillon, milk, infusion of malt, eggs, potato. It shows a preference for slightly acid media (p. 132). The growth develops slowly and shows a preference to growth beneath the surface of less solid media. On potato it develops on the surface in characteristic layers. The specific form of growth is that of moss-like projections from a central body (Fig. 100). The color is at first grayish-white, then yellowish. As seen under the microscope, ray-like filaments are developed, which divide into branches. The ends are often swollen or club-shaped, and there are various enlargements along the body of the filament. Several varieties of favus have been discovered.

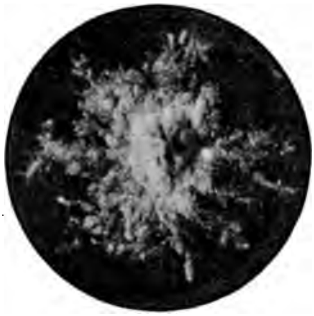

FIG. 100.—Five-month-old colony of favus on peptone-maltose agar; actual size.

Recently the Roentgen-ray treatment has been recommended for aborting the disease but this is not always practicable. Also specific vaccines have been tried but reports from their use are not yet satisfactory.

**Microsporon Furfur** (described by Eichstedt in 1846) —This organism, found in pityriasis versicolor, belongs to a group of fungi which, in contrast to the more parasitic fungi, favus and trichophyton, invades only the most superficial layers of the skin and does not give rise to any considerable pathological changes in the skin or hair. Although the vegetative elements of these fungi are much more numerous in the affected portions of the skin than is the case with the more parasitic species, they are not nearly so contagious as the latter.

By preference the organism attacks the chest, abdomen, back, and axillæ, less frequently neck and arms, while exceptionally it attacks also the face. The growth shows itself as scattered spots varying in color from that of cream-coffee to reddish-brown. The spots are readily scraped off and show fine lamellation or scaling. Occasionally the spots are confluent, and sometimes arranged in ring form like Herpes tonsurans.

In spite of their slight contagiousness this is one of the most frequent dermatomycoses. Although it is distributed widely over the earth, it is more frequently observed in southern than in northern countries.

Persons with a tender skin and a disposition to perspire freely are particularly affected by Pityriasis versicolor, and this is undoubtedly the only reason why the affection is so frequently observed in consumptives. Women are more frequently attacked than men, while children and old people are rarely affected.

The source of infection is unknown, since the absence of contagion has frequently been demonstrated. It seems likely that the spores of this fungus are so widely distributed that susceptible individuals are easily infected.

The arrangement of the fungus in the scales of the epidermis is characteristic. The short and thick curved hyphæ (7μ to 13μ long and 3μ to 4μ wide) surround large clumps of spores. The spores are coarse, doubly contoured (4μ to 7μ) and round. On staining with Ziehl's solution the spores are seen to contain deeply stained globules lying, in all probability, on the inner surface of the cell membrane. The rest of the protoplasm is but little stained, or not at all. One frequently finds that these globules are also seen free; what their nature is does not appear; they are not found in cultures, the freshly developed spores showing only a single globular mass of protoplasm possessing a fine blue luster.

Cultures are obtained with difficulty and the growths cannot be transferred for many culture generations. The growth is said to be most characteristic on potato which shows yellowish-white colonies within four or five days.

**Sporotricha.**—Another of the moulds classed with the *Fungi imperfecti* (family Mucedineæ) has been found to produce disease in man and some of the lower animals. The first variety described was by Schenk[1] in 1898. It was declared one of the sporotricha by F. Smith. In 1900 Hektoen and Perkins[2] described minutely a culture isolated from another case and gave the organism the name Sporotrix (Sporotrichon) schenkii.

Since the disease was made more thoroughly known by the studies of Beurmann and Gougerot[3] and others, many cases have been reported in various parts of the world, and several new species of sporotricha have been described. Beurmann and Gougerot first described the species so far most frequently reported, that is, Sporotrichon beurmanni, which is so similar to Sporotrichon schenkii that they are classed now by most observers as one species. So several of the other species described may prove on further study to be but one species.

Earlier the disease was often mistaken for tuberculosis or syphilis because of the similarity of the lesions which, in general, are slowly growing granulomata ending in degeneration. When it was found that the disease responds readily to treatment with potassium iodide and other iodine combinations, but not to mercury salts, it became comparatively easy to corroborate the diagnosis. In 1912 Ruediger[4] published a review of the cases reported to date. In the latter part of 1915 Meyer[1] reported that the disease is very common in certain parts of the United States, in domesticated animals, especially in horses, but that human beings do not readily contract the disease from them.

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[1] Johns Hopkins Hosp. Bull., 1898, p. 281.

[2] Jour. Exp. Med., 1900, v, 77.

[3] Traité des Sporotrichoses, Paris, 1912.

[4] Jour. Infect. Dis., 1912, xi, 193.

**Morphology.**—In the pus from the lesions only oval, highly refractile conidia or spores ($3\mu$ to $4\mu$ x $1.6\mu$ to $3\mu$) are seen and these generally sparingly. They may lie within the tissue cells. They are Gram-positive. In cultures these spores grow out into a branching irregularly septate mycelium (the hyphæ about $1\mu$ thick), and the new conidia are formed simply at the sides and ends of the hyphæ without definite fruiting organs. The conidia often occur in whorls. (Plate I, Fig. 5.)

**Cultivation.**—They grow readily on ordinary media, but better when it is slightly acid. Sabouraud's medium (p. 132) is most favorable. They grow both at room and at incubator (36° C.) temperature. At 20–25° C. growth is seen in about four days, and at 36° C. in about forty-eight hours. Minute, fluffy, snowflake-like colonies appear which slowly become brown or brown-black, and, when in a mass, convoluted. Gougerot found that by letting a drop of the pus flow over a glass slide and keeping in a moist atmosphere, the sprouting of the spores may be followed under the microscope in two or three days. Agglutination and complement fixation have been studied by Widal and Abrami (1908). Their results, which are given in the next paragraphs, await further corroboration.

**Agglutination.**—Homogeneous emulsions of the spores are made from cultures on Sabouraud's medium six to twelve weeks old. A large amount of the cultures is removed and rubbed until dry in a mortar. To this, drop by drop, is added several cubic centimeters of normal salt solution, while continuing to rub. The suspension is filtered through moistened filter paper. The filtrate contains only free spores which in positive cases agglutinate in from fifteen to sixty minutes in dilutions of 1 to 150 to 1 to 1800, generally 1 to 300 to 1 to 400. Agglutination with the sera of other mould and yeast infections may occur to less extent.

**Complement fixation** is said to be not specific for the different mould and yeast infections.

**Pathogenesis.**—Cultures or pus injected subcutaneously or intraperitoneally into lower animals such as mice, rats, and dogs produce granulomatous lesions similar to those found in man.

In man the lesions vary from superficial, non-ulcerative gummata to deep visceral abscesses. Sometimes the diagnosis is difficult to determine. According to Gougerot the following points help in differentiation:

1. Many lesions with general good health.

2. Lesions begin as indolent swellings which gradually become large gummata, on which one or several small ulcers with violet edges may appear. These ulcers discharge a thin, yellowish, shiny pus, and the

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[1] Jour. Am. Med. Assn., 1915, lxv, 579.

center gradually becomes cicatrized with a persistence of the abscess under the skin. Generally there is no adenitis.

3. Microscopic and cultural examinations reveal the organisms. Large quantities of the pus should be inoculated into each culture tube which should not be closed with rubber cap. The dry method should also be tried. Direct smears are not satisfactory.
4. Serum diagnosis may be tried.
5. Animal inoculations give positive results.
6. Treatment with potassium iodide. This produces a cure unless given very late in the disease or unless the mucous membranes are extensively affected. Some cases need treatment for two or three months before improvement is seen. Occasionally death occurs.

## PATHOGENIC YEASTS.

The definite biologic relationships of the monilia, the oidia, the torula and the yeasts, or saccharomycetes have not yet been determined (see page 28 for classification and general characteristics). The relations of the so-called coccidial organisms have not yet been settled. All we can do is to group them tentatively and wait for further investigations to help us place the pathogenic forms rightly. That we should still call all markedly budding forms blastomycetes and the diseases produced by them blastomycoses seems wiser than to drop these terms and call the infections "torula infections," "oidiomycosis" or "moniliosis" when we are not yet sure that the organisms producing them belong to the monilia, the torula or the oidia. But we are sure that these organisms all produce yeast-like forms in abundance which propagate chiefly by budding and hence may be called blastomycetes.

Stoddard and Cubler[1] (1916) conclude from a study of a culture obtained from a case of infection of the nervous system that the microörganism is a torula because it reproduces only by budding, it forms no mycelium and it produces no gaseous fermentation and they further conclude that because of apparently similar lesions in other cases reported the same germs might have been the cause in each. They give a table of what they consider the differential points between "torula infection, oidiomycosis (usually termed blastomycosis) and coccidioidal granuloma." More strains of these yeast-like organisms must be studied minutely in connection with the lesions produced before we can accept this grouping.

The pathogenic blastomycetes reported may be briefly summarized as follows:

Saccharomyces busse is the name given to a yeast isolated in 1894 by O. Busse from an abscess in the tibia of a thirty-one-year-old woman who died thirteen months after the first symptoms appeared. The autopsy showed numerous broken-down nodules on several of the bones, and in the lungs, spleen, and kidneys. The yeast was cultivated from all these foci.

Saccharomyces tumefaciens was isolated in 1895 by Curtis. The patient was a young man showing multiple tumors on the lips and neck having the gross appearance of softened myxosarcomata. This yeast is pathogenic for rats, mice, and dogs, only slightly so for rabbits, and not at all for guinea-pigs.

Various similar cases have since been described, a number of them becoming generalized and ending fatally. In generalized blastomycosis the lung seems frequently to be the seat of primary infection. Several cases of mixed infections have been reported as due to yeast and tubercle bacilli. Samplice[1] states that the lungs of tuberculous cattle often contain blastomycetes and other fungi and that the blastomycetes are indistinguishable from those isolated by him from human tumors. They seem to render the tuberculous infection more virulent.

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[1] Torula Infection in Man. Monograph 6 Rockefeller Institute, Jour. Med. Res., 1916, New York.

The cases described first by Rixford and Gilchrist as coccidiosis due to "Coccidioides imitis"—thought to be a protozoön—may be classed here, since Ophüls, Oliver, and others have shown that the "coccidium" forms hyphæ and elliptical forms on culture. Cummins and Sander[2] have shown that cultures from fresh tissue grow slowly (appearing in from seven to twelve days in primary cultures) but with ease on the usual laboratory media. The growth is more mould-like than yeast-like, except on potato, when yeast-like cells are produced, though no budding has been seen. Dogs, rabbits, and guinea-pigs are susceptible to the fungus and show lesions similar to those in human beings. The disease has been called coccidioidal granuloma. Zinsser[3] isolated an organism similar to those described by Ophüls and Oliver from an abscess of the back. Brown and Cummins[4] state, after an extensive study of "coccidiodal" disease, that the species causing this group of cases is distinct from that caused by the definitely budding type of yeast.

A typical case of systemic blastomycosis, reported by Fontaine, Hasse, and Mitchell[5] is accompanied by very good illustrations of tissue sections. Fig. 101 shows the characteristic microscopic appearance of the lung lesion.

Several cases of ophthalmia have been described as due to yeasts. Recently Jackson[6] and others have added to the reports of cases of eye infection by yeasts. Even "trachoma" has been said to be caused by this organism.

Buschke isolated a yeast from a cervical discharge in which no gonococci were present. The yeast was pathogenic for guinea-pigs.

In 1895 Tokishige reported an epidemic quite common among horses in Japan, known as "Japanese worm," "benign worm," or "pseudoworm," which is caused by one of the saccharomyces. This disease begins in the skin in the form of hard, painless nodules from the size of a pea to that of a walnut. These break down and give rise to gradually extending ulcers. Pure cultures of the saccharomyces are pathogenic chiefly for horses, not for rabbits, guinea-pigs, or hogs. In the districts where the disease prevails among horses it is also frequently seen in cattle.

Shortly after Tokishige's publication a similar disease occurring in horses in Italy and southern France was identified as being caused by saccharomyces. Cultures of this yeast, however, differ somewhat from that obtained in Japan, so that Busse is inclined to regard the two as two different species of blastomycetes.

Kartulis, in Alexandria, described about a hundred cases of a skin affection occurring in the gluteal regions of men and characterized by an elongated finger-like swelling, which breaks and emits a purulent discharge, forming an unhealed sinus. In the discharge and surrounding tissues are numerous blastomycetes which Kartulis after cultivation and study considered a variety of the ordinary fermenting yeast (Saccharomyces cerebisiæ Hansen). The cases were cured by excising the growth.

Kessler[7] reported a skin lesion in an infant (Fig. 102) probably due to a similar blastomycete, since the lesions healed after treatment with potassium iodide. The description of the yeast isolated is too incomplete to identify it.

Some years ago the attempt was made to connect the development of cancerous growth with blastomycetes. This was due in a measure to a certain similarity between the yeasts and the cell inclusions or so-called "parasites" of cancer, and further, to the fact that when yeasts are injected into the animal body tumor-like nodules are often developed at the site of inoculation and in the internal organs. But these nodules are not tumors in the pathological sense of the term, but merely masses of blastomycetes mixed to a very variable degree with inflammatory tissue proliferations.

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[1] Annals d'Igiene. 1918, xxviii, 41.

[2] Jour. Med. Res., 1916, xxxv, 243.

[3] Proc. New York Path. Soc., 1907.

[4] Arch. Int. Med., 1915, xv, 608 (with bibliography).

[5] Ibid., 1909, iv, 101.

[6] Jour. Am. Med. Assn., 1915, lxv, 23,

[7] Ibid., 1907, xlix, 550.

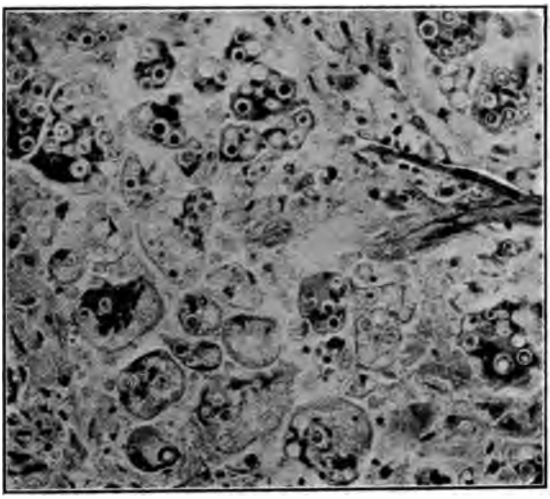

FIG. 101.—Section of lung. × 150; blastomycetes in large syncytial cell masses. (From Fontaine, Hasse, and Mitchell.)

*Thrush* (*Oöspora, Oïdium Albicans, Monilia Albicans*) (Fig. 103).—Thrush, as is well known, occurs most frequently in the oral mucous membrane of infants during the early weeks of life. It may also be found as a mycosis in the vagina, especially of pregnant women. In rare cases the disease attacks adults, and especially those whose system has been undermined by other diseases, such as diabetes, typhoid, etc. A few cases are recorded in literature in which this

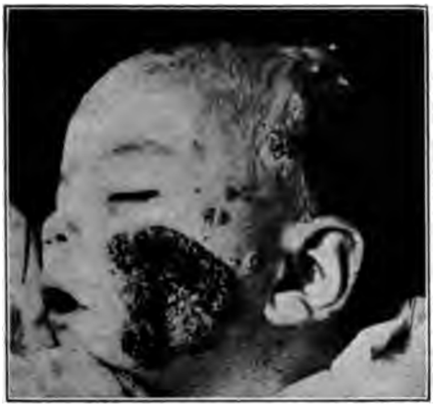

FIG. 102.—Blastomycosis in infant aged eight months, showing lesion on left cheek. (Kessler.)

fungus has given rise to constitutional disease. In these cases autopsy has shown abscesses in various parts of the body, such as the lungs, spleen, kidney and brain.

The tropical disease called *sprue* has been said by Baker to be due to Monilia albicans. Ashford[1] thinks this disease is caused by a distinct species of monilia, Monilia psilosis.

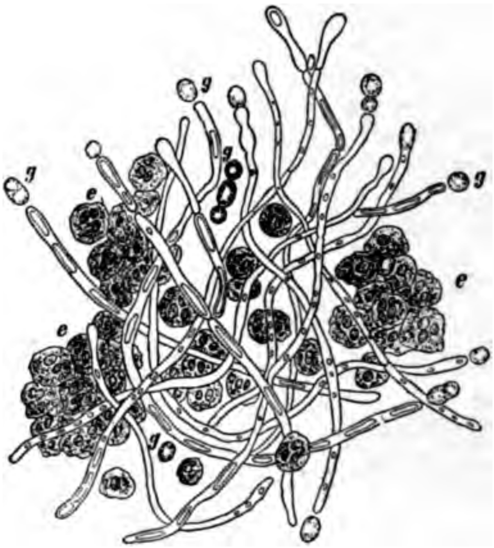

FIG. 103.—Inflammation of cornea by thrush (Oïdium albicans), *g*, conidia; *e*, pus cells. (From Plaut, in Kolle and Wassermann.)

In the lesions of the disease as well as in cultures this fungus appears as both a yeast and a mycelium. It thus seems to stand between the true moulds and the yeasts. The yeast cells are oval in form, about 5 μ to 6 μ long and 4 μ wide, and can in no way be distinguished from other yeast cells either by their appearance or their method of propagation. The threads of the mycelium vary markedly in length and thickness, and show all intermediate forms between a typical and a budding mycelium. Cultures are easily obtained on artificial media, especially on those favorable for yeasts (p. 132).

Michel[2] has reported favorable results in tropical sprue by treating with a vaccine made from Monilia psilosis strains.

Thrush is not much influenced by acids or alkalis. On the other hand, it is very susceptible to the common disinfectants, especially salicylic acid, corrosive sublimate, phenol, iodine, etc. This fact is made use of in local treatment.

The important part played by yeasts in certain industries is treated in Part III.

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[1] Etiology of Sprue, Am. Jour. Med. Sc., 1917, cliv, 157.

[2] Monilia Vaccine in Sprue, Jour. Inf. Dis., 1918, xxii, 53.

# CHAPTER XVI.

# THE PYOGENIC COCCI.

## THE STAPHYLOCOCCI (MICROCOCCI).

PRACTICALLY all microörganisms, when inoculated into the animal body, have been shown by experiment to induce, under certain conditions, the formation of pus by their products. While this has been demonstrated, the researches of bacteriologists show that only a few species are usually concerned in the production of acute abscesses in man. Of these the two most important, by reason of their frequent occurrence and pathogenic power, are Staphylococcus aureus and Streptococcus pyogenes. These two organisms are often found in the same abscess; thus, Passet, in 33 cases of acute abscess, found Staphylococcus aureus alone in 6, aureus and albus associated in 11, albus alone in 4, albus and citreus in 2, Streptococcus pyogenes alone in 8, albus and Streptococcus in 1, and albus, citreus, and Streptococcus in 1. The staphylococcus is likely to enter as a mixed infection into most infections due to other bacteria, and is almost always met with in inflammations of the skin and mucous membranes or in cavities connected with them.

Staphylococci were first obtained from pus by Pasteur in 1880. In 1881 Ogston[1] showed that they frequently occurred in abscesses, and in 1884 Rosenbach[2] fully demonstrated their etiological importance in circumscribed abscesses, osteomyelitis, etc. Of the staphylococci those producing yellow and white pigments are by far the most important since they are the pathogenic varieties.

**The Staphylococcus (Pyogenes) Aureus.**—The *Staphylococcus aureus* is one of the commonest pathogenic bacteria, being usually present in the skin and mucous membranes, and is the organism most frequently concerned in the production of acute, circumscribed, suppurative inflammations.

**Morphology.**—Small, spherical cells, having an average diameter of $0.7\mu$ to $0.9\mu$, occurring solitary, in pairs as diplococci, in short rows of three or four elements, or in groups of four, but most commonly in irregular masses, simulating clusters of grapes; hence the name staphylococcus. (See Fig. 104.)

**Staining.**—It stains quickly in aqueous solutions of the basic anilin colors and with many other dyes and is not decolorized by Gram's method. When slightly stained each sphere frequently is seen to be already dividing into two hemispherical bodies.

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[1] British Med. Jour., 1881, i, 369.

[2] Microörganismen bei Wundinfektion, Wiesbaden, 1884.

**Biology.**—The Staphylococcus (pyogenes) aureus is a non-motile, aërobic, facultative anaërobic micrococcus, growing at a temperature from 8° to 43° C., but best at 25° to 35° C. The staphylococci grow readily on all the common laboratory media, such as milk, bouillon, nutrient gelatin, or agar. A slightly alkaline reaction to litmus is best for the growth of the staphylococci, but they also grow in slightly acid media.

**Cultivation.**—**Growth in Nutrient Bouillon.**—The growth of the staphylococcus is rapid, reaching about 500,000,000 per c.c. at the end of twenty-four hours at 30° C. The bouillon is cloudy and frequently has a thin pellicle. Later a slimy sediment forms. The odor is disagreeable. In peptone-water, growth occurs with indol production.

**Growth on Gelatin.**—Grown on gelatin plates at room temperature, within forty-eight hours, punctiform colonies develop, which when examined under a low-power lens, appear as circular disks of a pale brown color, somewhat darker in the center, and surrounded by a smooth border. The colonies grow rapidly. The appearance of the growth is most characteristic. Immediately surrounding the colonies, which are of a pale golden yellow color, there is a pitting of the surface of the gelatin, due to its liquefaction. By suitable light a number of these shallow depressions with sharply defined outlines may be seen on the gelatin plate, having a diameter of from 5 to 10 mm., in the centers of which lie the yellow colonies. Later the liquefaction becomes general, the colonies running together. In stab cultures in gelatin a white confluent growth at first appears along the line of puncture, followed by a funnel-shaped liquefaction of the medium, which rapidly extends to the sides of the test-tube. At the end of two days the yellow pigmentation begins to form, and this increases in intensity for eight days. Finally, the gelatin is completely liquefied, and the staphylococci form a golden-yellow or orange-colored deposit at the bottom of the tube. Under unfavorable conditions the Staphylococcus aureus gradually loses its ability to make pigment and to liquefy gelatin. The liquefication is due to a ferment called gelatinase formed by the staphylococci. It may be separated from the cocci by filtration (Loeb[1]).

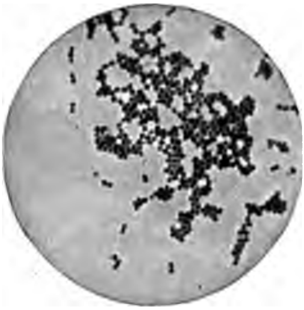
FIG. 104.—Staphylococcus. × 1100 diameters.

**Growth on Agar.**—In streak and stab cultures on agar a whitish growth is at first produced, and this at the end of a few days becomes a faint to a rich golden yellow on the surface. The yellow pigmentation is produced only in the presence of oxygen; colonies formed at the bottom of a stab culture or under a layer of oil remain white.

**Milk.**—Milk is coagulated at the end of from one to eight days.

**Potato.**—The staphylococci grow readily on potato and produce abundant deep colored pigment.

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[1] Centralbl. f. Bakt., 1902, xxxii, 471.

**Growth on Löffler's Solidified Blood Serum.**—Growth vigorous, with fairly good pigment production. Some varieties slowly liquefy the serum.

**Growth on Blood Agar.**—If nutrient agar to which a little animal blood has been added is streaked with staphylococci there appears, at the end of twenty-four hours at 35° C., about the growth a clear zone, owing to the hemolytic effect of the staphylococcus products.

**Acids Produced.**—In media containing carbohydrates there is, as a result of the growth of the Staphylococcus aureus, a production of acid in considerable quantities, consisting chiefly of lactic, butyric, and valerianic acids. These acids have been supposed to play a part in the production of pus, in which, according to some observers, they are often present. No gas is formed.

**Resistance.**—The staphylococcus is distinguished from most other non-sporebearing pathogenic bacteria by its greater power of resistance to outside influences, desiccation, etc., as well as to chemical disinfectants. Cultures of the Staphylococcus pyogenes in gelatin or agar retain their vitality for a year or more. Suspended in water its thermal death-point varies with different cultures and averages about two hours at 50° C., one-half hour at 60° C., ten minutes at 70° C., and five minutes at 80° C. Upon silk threads and in media rich in organic matter its resistance is greater, but subjected to 80° C., for thirty minutes or boiling for two minutes it is almost surely killed. Cold has but little effect. Thirty per cent. of the organisms remained alive after being subjected by us to freezing in liquid air for thirty minutes. These are average figures. Some cultures are more resistant than others.

They are quite resistant to direct sunlight and drying. Dried pus contains living staphylococci for weeks and even months, and they can be found alive in the fine dust of the air in living rooms and operating rooms.

Resistance to chemicals is given in Part III.

**Pigment Formation.**—Pigment formation, already described, is considered within limits a species characteristic. Thus different strains of Staphylococcus aureus produce a pigment varying from a pale brown to a deep golden yellow. It usually becomes less intense upon prolonged cultivation. The pigment is classed as a lipochrome (Schneider[1]). It is soluble in alcohol, chloroform and ether.

In order to test the amount of color produced, Winslow and Rogers recommend the following method: A portion of the growth is removed on a loop needle and spread out on white drawing paper with a rough surface. After drying at room temperature the color is compared with a standard color chart.

**Pathogenesis.**—The pathogenic effect of the Staphylococcus (pyogenes) aureus on test animals varies considerably, according to the mode of application, the virulence of the special culture employed and the species of animal used. In man a simple rubbing of the surface of the unbroken skin with pus from an acute abscess is frequently sufficient to produce a purulent inflammation, and the introduction of a few germs from a septic case into a wound may lead to a fatal pyemia. These conditions can only be reproduced in lower animals with difficulty, and by the inoculation of large quantities of the culture. Small subcutaneous injections, or the inoculation of open wounds in mice, guinea-pigs, and rabbits, are commonly without result; occasionally abscess formation may follow at the point of inoculation, which usually ends in recovery. The pus-producing property of the organism is exhibited in proportion to the virulence of the culture employed. Slightly virulent cultures, which constitute the majority of those obtained from pus taken from the human subject, when injected subcutaneously in large quantities (several cubic centimeters of a fresh bouillon culture) into rabbits or guinea-pigs, give rise to local pathological lesions—acute abscesses. When virulent cultures are used—usually those recently isolated from human infections —0.5 c.c. of a fresh bouillon culture is sufficient to produce similar results. The abscesses generally heal without treatment; sometimes the animals die from marasmus in consequence of the suppurative process. In intraperitoneal inoculations the degree of virulence of the culture employed is still more evident in the effects produced. The animals usually die in from two to nine days. The most characteristic pathological lesions are found in the kidneys, which contain numerous small collections of pus, and under the microscope present the appearances resulting from embolic nephritis. Punctiform, whitish-yellow masses of the size of a pea are found permeating the pyramids. Many of the capillaries and some of the smaller arteries of the cortex are plugged up with thrombi, consisting of micrococci. Metastatic abscesses may also be observed in the joints and muscles. The micrococci may be recovered in pure cultures from the blood and the various organs; but they are not numerous in the blood and are often difficult to demonstrate microscopically. Intravenous inoculations of animals are followed by similar pathological changes. Orth and Wyssokowitsch first pointed out that injection of staphylococci into the circulation of rabbits, whose cardiac valves have previously been injured, produced ulcerative endocarditis. Subsequently Weichselbaum, Prudden, and Fränkel and Sänger obtained confirmatory results, thus establishing the fact that when the valves are first injured, mechanically or chemically, the injection into a vein of a pure culture of Staphylococcus aureus gives rise to a genuine ulcerative endocarditis. It has been further shown by Ribbert that the same result may be obtained without previous injury to the valves by injecting into a vein the staphylococcus from a potato culture suspended in water. In his experiments not only the micrococci from the surface, but the superficial layer of the potato were scraped off with a sterilized knife and mixed with distilled water and the successful result is ascribed to the fact that the little agglomerations of micrococci and infected fragments of potato attach themselves to the margins of the valves more readily than isolated cocci would do. Not infrequently, also, in intravenous inoculations of young animals there occurs a localization of the injected material in the marrow of the small bones. This may take place in full-grown animals when the bones have been injured or fractured. The experimental osteomyelitis thus produced has been demonstrated to be anatomically analogous to this disease in man. An increase in virulence of certain strains may be obtained by successive passage through susceptible animals.

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[1] Arb. a. d. bakt. Inst. Karlsruhe, 1891, i.

**Toxic Substances Produced.**—Filtrates of cultures contain toxic substances. Injected into the peritoneal cavity they excite peritonitis. Under the skin they produce infiltration or abscess formation. In the blood they injure both the red and white corpuscles.

Cultures of the staphylococcus, when sterilized by boiling and injected subcutaneously, produce marked positive chemotaxis and often local abscesses. Leber found also that sterilized cultures introduced into the anterior chamber of the rabbit's eye would bring about a fibropurulent inflammation, the cornea becoming infiltrated, and perforation alongside of the sclerotic ring finally taking place. This was followed by the formation of pus in the anterior chamber and recovery. These local changes follow the inoculation of small quantities only of the dead cultures; but when large amounts are injected into a vein or into the abdominal cavity, toxic effects are produced. The hemolytic effects of certain products of the staphylococcus have been studied. In cultures they can be detected about the third or fourth day of incubation and reach their maximum on the ninth to fourteenth day. Virulent staphylococci are more apt to produce this substance than the nonvirulent, but there is no definite rule.

The specific hemolysin, known as staphylolysin, is destroyed by heating for twenty minutes at 56° C. An antibody for this is formed by inoculating animals with culture filtrates. A substance called leukocidin is produced which injures leukocytes, it also stimulates the production of an antibody. The gelatin-liquefying enzyme—gelatinase—has already been mentioned.

**Occurrence in Man.**—The staphylococcus (*Staphylococcus aureus*) has been demonstrated not only in furuncles and carbuncles, but also in various pustular affections of the skin and mucous membranes—impetigo, sycosis, purulent conjunctivitis and inflammation of the lacrimal sac; in acute abscesses formed in the lymphatic glands, the parotid gland, the tonsils, the mammæ, etc.; in metastatic abscesses and purulent collections in the joints; in empyema, infectious osteomyelitis, ulcerative endocarditis, pyelonephritis, abscess of the liver, phlebitis, meningitis, etc. It is one of the chief etiological factors in the production of pyemia in the various pathological forms of that condition of disease. It is remarkable how many staphylococci may be present in the blood without a fatal result, if the source of infection is removed. We met with one case in which over 800 staphylococci were present in 1 c.c. of blood. A week later only 5 were found. The patient finally died from pneumonia, due to the pneumococcus.

A number of cases of staphylococcus pneumonia have been described as occurring among the troops during the recent war.[1]

Not all persons are equally susceptible to infection by the staphylococcus; those who are in a cachectic condition or suffering from constitutional diseases, like diabetes, are especially predisposed to infection. In healthy individuals certain parts of the body, as the back of the neck and the buttocks, are more liable to be attacked than others, with the production of furuncles, carbuncles, etc. In persons in whom sores are readily caused, in consequence of disturbances of nutrition, as in exhausting diseases, the micrococci settle at the points of least resistance. Such conditions are present in the bones of debilitated young children, in fractures, and in injuries in general.

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[1] **Chickering and Park**: Jour. Am. Med. Assn., 1919, lxxii, 617.

**Immunity.**—Rabbits have been rendered immune by means of inoculations with either dead or living cultures. Unless the inoculations are carefully made the animals frequently succumb. The staphylococci injected into an immunized animal are more rapidly taken up by the leukocytes than when injected into an untreated animal. (See Opsonins, p. 218.)

A serum having some protective power has also been elaborated.

Hiss claimed good results from the use of leukocyte extracts in animals infected with *Staphylococcus aureus.*

**Therapeutic Use of Vaccine.**—The treatment of abscesses, boils, and other localized staphylococcus infections as well as general infections by injections of repeated doses of vaccine is considered in Part III.

**Staphylococcus (Pyogenes) Albus.**—It is morphologically identical with the *Staphylococcus (pyogenes) aureus,* and may be the same organism which has lost the property of producing pigment. On the average it is somewhat less pathogenic and seldom produces pyemia or grave infections. The surface cultures upon nutrient agar and potato have a milk-white color. Its biological characters are not to be distinguished from the *Staphylococcus aureus.*

The majority of bacteriologists agree with Rosenbach,[1] that the aureus is found at least twice as frequently in human pathological processes as the albus.

**Staphylococcus Epidermidis (Albus) (Welch).**—Probably identical with the *Staphylococcus (pyogenes) albus.* With reference to this micrococcus, Welch says: "So far as our observations extend—and already they amount to a large number—this coccus may be regarded as nearly, if not quite, a constant inhabitant of the epidermis. It is now clear why I have proposed to call it the *Staphylococcus epidermidis albus.* It possesses such feeble pyogenic capacity, as is shown by its behavior in wounds as well as by experiments on rabbits, that the designation *Staphylococcus pyogenes albus* does not seem appropriate. Still I am not inclined to insist too much upon this point, as very probably this coccus—which has hitherto been unquestionably identified by others with ordinary *Staphylococcus pyogenes albus* of Rosenbach—is an attenuated or modified form of the latter organism, although, as already mentioned, it presents some points of difference from the classical description of the white pyogenic coccus."

According to Welch, this coccus differs from the *Staphylococcus albus* in the fact that it liquefies gelatin more slowly, does not so quickly cause coagulation in milk, and is far less virulent when injected into the circulation of rabbits. It has been shown by the experiments of Bossowski and of Welch that this microörganism is very frequently present in clean wounds, and that usually it does not materially interfere with the healing process, although sometimes it appears to cause suppuration along the drainage tube, and is the common cause of "stitch abscesses."

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[1] Microörganismen bei Wundenfektion, Wiesbaden, 1884.

**Staphylococcus (Pyogenes) Citreus.**—Isolated by Passet (1885) from the pus of acute abscesses, in which it is occasionally found in association with other pyogenic cocci. It is distinguished from the other species only by the formation of a lemon-yellow pigment.

**Other Staphylococci.**—Other varieties have been occasionally met with which differ in some respects from the typical varieties. This difference may be in the fact that they liquefy gelatin more slowly or not at all, or in pigment formation, or in agglutination, or in still other respects. None of these varieties seem to be of importance.

## THE MICROCOCCUS TETRAGENUS.

This organism was discovered by Gaffky (1881). It is not infrequently present in the saliva of healthy individuals and in the sputum of consumptive patients. In sputum it is sometimes an evidence of mouth contamination rather than lung infection. It has been observed repeatedly in the walls of cavities in pulmonary tuberculosis associated with other pathogenic bacteria, which, though playing no part in the etiology of the original disease, contribute, doubtless, to the progressive destruction of the lung. Its pyogenic character is shown by its occasional occurrence in the pus of acute abscesses. Its presence has also been noted in the pus of empyema following pneumonia.

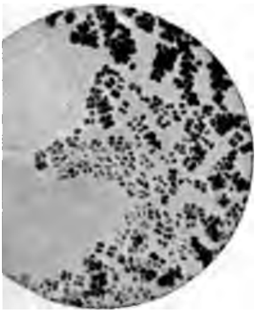

FIG. 105.—Micrococcus tetragenus. Stained with methylene blue. × 800 diameters.

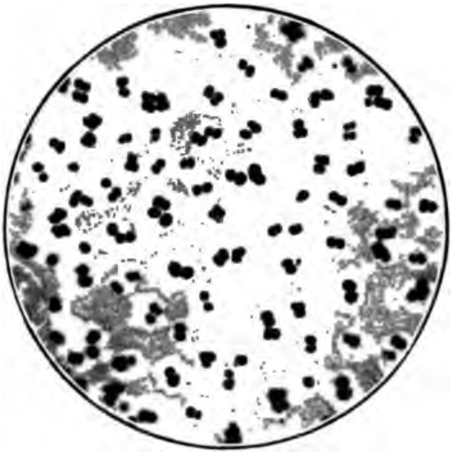

FIG. 106.—Micrococcus tetragenus from peritoneal fluid. Stained with fuchsin. (Fränkel.) × 1000 diameters.

**Morphology.**—Micrococci having a diameter of about 1$\mu$, which divide in two planes, forming tetrads, and bound together by a transparent, gelatinous substance, enclosing the cell like a capsule. In cultures the cocci are seen in various stages of division as large, round cells in pairs of oval elements, and in groups of three and four (Figs.

105 and 106). When the division is complete they remind one of sarcinæ in appearance, except that they do not divide in three directions and are not built up like diminutive cotton bales (see also Plate II, Fig. 4, and Plate III, Fig. 11).

**Staining.**—This micrococcus *stains* readily with the ordinary anilin dyes; the transparent gelatinous envelope is only feebly stained. It is not decolorized by Gram's method.

**Biology.**—The growth of this micrococcus is slow under all conditions. It grows both in the presence and absence of oxygen; it grows best from 35° to 38° C., but may be cultivated also at the ordinary room temperature—about 20° C.

**Growth on Gelatin.**—On gelatin plates small, white colonies are developed in from twenty-four to forty-eight hours, which, when examined under a low-power lens, are seen to be spherical or lemon-shaped, grayish-yellow disks, with a finely granular or mulberry-like surface, and a uniform but somewhat roughly dentated border. When the deep colonies push forward to the surface of the gelatin they form white, elevated, drop-like masses, having a diameter of 1 to 2 mm. In gelatin stick cultures the gelatin is not liquefied.

**Growth on Agar and Blood Serum.**—The colonies appear as small transparent, round points, which have a grayish-yellow color and are slightly elevated above the surface of the medium.

**Pathogenesis.**—Subcutaneous injections of a culture of this micrococcus in minute quantity is usually fatal to white mice. The micrococci are found in comparatively small numbers in the blood of the vessels and heart, but are more numerous in the spleen, lungs, liver, and kidneys. Intraperitoneal injections given to guinea-pigs and mice are followed by purulent peritonitis, beautifully formed cocci in groups of four being obtained in immense numbers from the exudate. Rabbits and dogs are not affected by large doses of a culture subcutaneously or intravenously administered.

In man it is generally non-pathogenic, except in the conditions already cited. It has been obtained by us as the only organism in a case of chronic conjunctivitis.

The serum from immunized cases has not been used therapeutically in human infection. Vaccines may be employed as with staphylococci.

## THE STREPTOCOCCI.

The streptococci in their relation to human infection outweigh in importance most other disease-producing organisms. Under this name must be included not only the streptococci which excite inflammation in man, but all non-motile, non-sporebearing spherical bacteria which divide, as a rule, in one plane only and remain attached in longer or shorter chains. There appear to be many saprophytic and non-pathogenic parasitic varieties, as well as many races of pathogens. Therefore from the practical standpoint of the clinician it is extremely important to be able to separate the pathogenic forms from the non-pathogenic forms and to determine the relationship between the pathogenicity of the former and their other characteristics. But, owing to the variations in morphology, cultural characteristics and virulence of this group of bacteria it has so far been impossible to give satisfactory differentiation of varieties in the group. The relation between pathogenicity and other characteristics is not clear-cut. The relation of the pneumococcus to the group has not been fully decided. The work of Rosenow on the transmutation of streptococci and pneumococci still calls for confirmation. There have been many attempts to classify this group of bacteria especially since the discovery of pathogenic forms. The first pathogenic streptococci were discovered by Koch in stained sections of tissue attacked by septic processes, and by Ogston in the pus of acute abscesses (1881). Pure cultures were obtained by Fehleisen (1883) from a case of erysipelas. The cultural and pathological characters were studied by him and it was shown to be capable of producing erysipelas in man. Rosenbach (1884) and Krause and Passet (1885) isolated the streptococcus from the pus of acute abscesses and gave it the name of *Streptococcus pyogenes*. The first classification was thus based chiefly on pathogenicity. It was first thought that the streptococci of erysipelas, of acute abscesses, of septicemia, of puerperal fever, etc., belonged each to a different species, because they seemed to possess differences in their biological and pathological characteristics, according to the source from which they were obtained. But it was later thought that the slight differences among the majority of streptococci from these diseases were but acquired variations of organisms derived from the same species.

The first grouping of the pathogenic forms on biochemical characterists was made by Schottmüller in 1903. His three types were: (1) *Streptococcus pyogenes* or *erysipelatus*, which shows hemolysis on blood agar plates; (2) *Streptococcus mitior* or *viridans*, which produces a green halo about the colonies on blood agar plates, and (3) *Streptococcus mucosus* which produces a mucoid growth and a dark green zone. Park and Williams (1905) and others since then, showed that Streptococcus mucosus should be placed with the pneumococci under the common name pneumococcus mucosus. Gordon (1903) and Andrews and Horder (1906), using nine test substances, chiefly carbohydrates, on over a thousand strains, divided streptococci from various sources into nine groups. This work has been for the most part corroborated, but there has been much unfavorable criticism of such a classification based only on biochemical reactions. Serological reactions while encouraging, have not yet given clear-cut results. Lyall (1915) repeated the work with the carbohydrates and tried to show the relationship between the characteristic carbohydrate reaction and the reactions on erythrocytes, but evidence tends to show that the production of methemoglobin is not clear cut enough to be used as a primary basis for classification. Smith and Brown's[1] grouping (1915) based on erythrocyte reaction and morphology of colonies is a very broad grouping, and does not show the relationship of erythrocyte plate reaction to erythrocyte tube reaction. They attempted to show relationship between these characteristics and carbohydrate reactions as well as pathogenicity for rabbits. Holman's[1] classification is also based on erythrocyte and carbohydrate reactions and their correlation with pathogenicity.. Avery and Cullen[2] state that the hydrogen ion concentration at which human strains of hemolytic streptococci cease to grow ($P_h$ 5.2 to 5.0) is different from that of bovine strains ($P_h$ 4.5 to 4.3). Until we can show the relationship of specific serum reactions[3] to other characteristics we cannot do more than accept tentatively some broad practical classifications such as that of Smith and Brown or Blake,[4] and work at a standardization of methods for making the tests such as Brown[5] has been doing for plate reactions on erythrocytes and Lyall for tube reactions on the same cells. The following outline is an example of a general practical classification similar to Blake's.

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[1] Jour. Med. Res., 1915, xxxi, 455.

### STREPTOCOCCI.

Gram-positive chain-forming cocci, insoluble in bile, ferment dextrose and maltose, but usually not inulin.

Action on red blood cells:

I. Produce definite hemolysis. Streptococcus pyogenes (Rosenbach). Synonyms: St. hemolyticus[6] (Rolly). "Type β (*beta*)" (Smith and Brown). St. hemolysans (Blake) and others.
    Sugars: Most strains ferment saccharose, lactose and salicin, but not maltose. (See Brown's Table II.[4])

II. Produce no definite hemolysis.
  A. Produce methemoglobin. Streptococcus viridans must be placed here lacking comparative studies (Schottmüller and others).
  Synonyms: "Type α (*alpha*)" (Smith and Brown's).
    Sugars: May be divided into three groups according to action on mannit and lactose.
      1. Mannit − lactose + = St. buccalis (Blake), including St. mitis and St. salivarius of Andrews and Horder.
      2. Mannit + lactose + = St. fecalis. (Andrews and Horder.)
      3. Mannit − lactose − = St. equinus. (Andrews and Horder.)
  B. Produce no methemoglobin. St. anhemolyticus (Zangernast).
    Synonyms: St. saprophyticus (Mandelbaum) and "Type λ (*gamma*)" (Brown).
    Sugars: Too few strains have been studied with sugars to make a general grouping. (See Brown's Table II.[4])

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[1] Jour. Med. Res., 1916, xxxiv, 377 (with bibliography).

[2] Jour. Exp. Med., 1919, xxix, 215.

[3] Dochez, Avery and Lancefield have just published (Jour. Exp. Med., 1919, xxx, 179) studies showing that at least four biologic types exist among strains of St. hemolyticus from humans as shown by agglutination and protection.

[4] Jour. Med. Res., 1917, xxxvi, 99.

[5] Brown, J. H.: Monograph No. 9 of the Rockefeller Institute of Medical Research. 1919.

[6] The terms hemolyticus and hemolysans are more inclusive than the term pyogenes, since several varieties have been described that hemolyse red blood cells and that have fermentative characteristics other than those accepted for St. pyogenes. (See Holman's Table.)

**Streptococcus Pyogenes.**—Under this heading are included all those streptococci that produce hemolysis and ferment dextrose, lactose, saccharose, and salicin but that do not ferment mannit or inulin.

**Morphology.**—The cocci, when fully developed are spherical or oval. They have no flagella or spores. They vary from 0.4$\mu$ to 1$\mu$ in diameter.

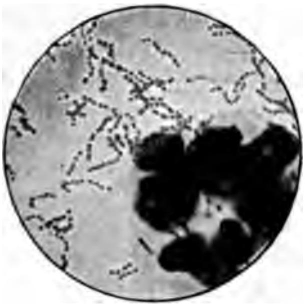

FIG. 107.—Streptococci in peritoneal fluid, partly enclosed in leukocytes. × 1000 diameters.

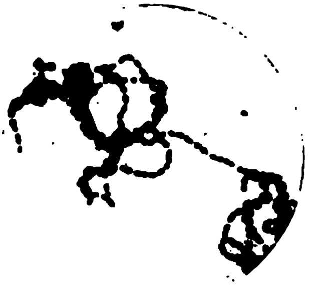

FIG. 108.—Streptococcus growing in long chains in bouillon culture. × 1000 diameters.

They vary in dimensions in different cultures and even in different parts of a single colony. They multiply by binary division in one direction only, forming chains of eight, ten, twenty, and more elements, being, however, often associated distinctly in pairs. On solid media the cocci occur frequently as diplococci, but usually they grow in longer or shorter chains. Frequently certain cocci exceed their fellows greatly in size, especially in old cultures, when they may be considered the result of involution processes. Hueppe called these arthrospores. Some varieties have distinct capsules when growing in the blood and in blood-serum media.

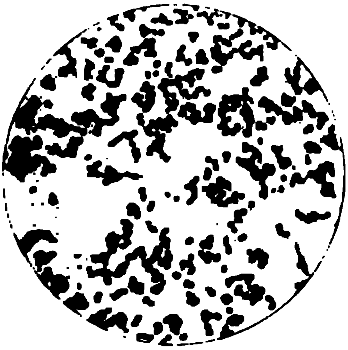

FIG. 109.—Streptococci from solidified serum culture appearing mostly in diplococci. × 1000 diameters.

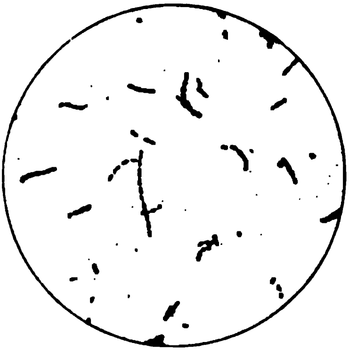

FIG. 110.—Streptococci in throat exudate smeared on cover-glass. × 1000 diameters.

**Staining.**—They *stain* readily by anilin colors. Most varieties give a positive reaction by Gram's method. Some species, mostly saprophytic, growing in short chains are negative to Gram's stain. Hemolytic strains are not usually as Gram-positive as are viridans strains.

**Biology.**—Streptococci of the pyogenes type grow readily in various liquid and solid culture media. The most favorable temperature for their development is from 30° to 37° C., but they multiply to some extent at ordinary room temperature—18° to 20° C. They are facultative anaërobes, growing both in the presence and absence of oxygen.

**Cultivation.—Growth on Gelatin.**—Tubes of gelatin which have been inoculated with certain strains by puncture with a platinum needle show on the surface, little growth beyond the point of entrance. In the depth of the gelatin on the second or third day a distinct, tiny band appears with granular edges or made up of granules. These granules may be very fine or fairly coarse. They are nearly translucent, with a whitish, yellowish, or brownish tinge. With characteristic cultures the gelatin is *not liquefied.*

**Growth on Agar.**—On agar plates the colonies are visible after twelve to thirty hours' growth at 37° C., and present a beautiful appearance when magnified sufficiently to see the individual cocci in the chain. The colonies are small, not averaging over 0.5 mm. in diameter (pinhead). From different sources they vary in size, thickness, mottling, color, and in the appearance of their borders. The streptococcus growing in short chains in bouillon shows but little tendency to form true loops, but rather projecting rows at the edges of the colonies, while those growing in long chains show beautiful loops, which are characteristic of this organism.

**Growth in Bouillon.**—Most streptococci of this type grow well in slightly alkaline bouillon at 37° C., reaching their full development within thirty-six to forty-eight hours. Those which grow in long chains usually give an abundant flocculent deposit and leave the liquid clear. The deposit may be in grains, in tiny flocculi, in larger flakes, or in tough, almost membranous masses, the differences depending on the strength of union between the pairs of cocci in the chains. Some of the streptococci growing in long chains, however, cause the broth to become cloudy. This cloudiness may be only temporary or it may be lasting. Those growing in short chains, as a rule, cloud the broth, this cloudiness remaining for days or weeks. A granular deposit appears at the bottom of the tube. An addition of 0.5 to 1 per cent. glucose aids the development of streptococci, but the acid produced tends later to hasten their death and make them lose virulence. A trace of calcium aids the growth as it neutralizes some of the acids produced. This is best added as a piece of marble.

**Growth in Ascitic or Serum Bouillon.**—The development in this, which is one of the best mediums for the growth of all streptococci, is more abundant than in plain bouillon. The liquid is usually clouded, and a precipitate occurs after some days, the fluid gradually clearing. The addition of blood serum frequently causes streptococci, growing in short chains in nutrient bouillon, to produce long chains. The reverse is also true, and in the blood all forms are usually found, some, at least, being diplococci or in short chains.

**Effect on Inulin.**—This is not fermented by varieties of the pyogenes type.

**Growth on Solidified Blood Serum.**—This is also an excellent medium for the streptococcus. Tiny, grayish colonies appear twelve to eighteen hours after inoculation.

**Growth in Milk.**—Streptococcus pyogenes strains grow well in milk. As a rule when growth is luxuriant a marked production of lactic acid with coagulation of the casein occurs.

**Development of Hemolytic Substances.**—Hemolysis is demonstrated within blood agar plates or in fluid containing blood in test-tubes. The plate method is as follows: If 1 c.c. of fresh or defibrinated blood is added to 6 c.c. of melted agar at 40° to 45° C., well shaken, inoculated with characteristic streptococci and poured into a Petri dish there will appear in twelve to twenty-four hours tiny colonies surrounded by clear zones of about ¼ to ⅓ inch in diameter.

The tube method is used for quantitative determination of hemolysis. The titration is made by adding decreasing amounts of a definite culture (eighteen-hour 2 per cent. peptone ascitic broth culture, according to Lyall), to a constant quantity of washed red blood cells (1 c.c. of a 5 per cent. suspension of sheep's red cells: Lyall). The tubes are incubated in water-bath at 37° C. for one hour and readings are then made.

Pneumococci and many streptococci grouped as *Streptococcus viridans,* which occur together with hemolytic forms in the throat, lungs, and elsewhere, on the other hand, produce only narrow zones of a green pigment. Anthony in our laboratory has found that from a streptococcus producing abundant hemolytic substances strains may be obtained, by selecting certain colonies, which fail to make them. She has not been able to obtain from strains producing in first cultures the green pigment only any strains producing hemolytic substances. Rosenow, on the other hand, claims that he can readily change certain non-hemolytic strains into hemolytic strains.

**Duration of Life Outside of the Body.**—This is not, as a rule, very great. When dried in blood or pus, however, they may live for several months at room temperature, and longer in an ice-chest, and in gelatin and agar cultures they live for from one week to three months. In order to keep streptococci alive and vigorous, it is best to transplant them frequently. They may be kept alive for a long time in semisolid agar stick cultures at room temperature or in serum or ascitic fluid bouillon in small sealed glass tubes in the ice-chest.

**Resistance to Heat and Chemicals** is given under Disinfection, Part III.

**Pathogenesis.**—The majority of test animals are not markedly susceptible to infection by streptococci directly from human beings. White mice and rabbits are the most susceptible, and these animals are therefore usually employed for experimentation. Streptococci, however, differ greatly in the effects which they produce in inoculated animals, according to their animal virulence. The most virulent when injected in the minutest quantity into the circulation or into the subcutaneous tissue of a mouse or rabbit, produce death by septicemia. Those of somewhat less virulence produce the same result when injected in considerable quantities. Those still less pathogenic produce septicemia, which is mild or severe, when injected into the circulation; but when injected subcutaneously, they produce abscess or erysipelas. The remaining streptococci, unless introduced in quantities of 20 c.c. or over, produce only a slight redness, or no reaction at all, when injected subcutaneously, and little or no effect when injected directly into the circulation.

One important fact that experience teaches us is that those streptococci which are the most dangerous are those which have come immediately from septic conditions, and the more virulent the case the more virulent the streptococci are apt to be for animals of the same species. There seems also to be a strong tendency for a streptococcus to produce the same inflammation, when inoculated, as the one from which it was obtained; for example, streptococci from erysipelas tend to produce erysipelas, from septicemia to produce septicemia, etc. This question of the localization of streptococci in the special tissue from which they were obtained, that is, their elective localization, has been much studied, especially by Rosenow. The relationship of this apparent power to true mutations is still undecided.

**Occurrence in Man.**—Hemolytic streptococci have been found to be a primary cause of infection in the following diseases: Erysipelas, circumscribed and extensive acute abscesses, impetigo, cellulitis (circumscribed as well as diffused), sepsis, puerperal infection, acute peritonitis, angina, bronchopneumonia, periostitis, osteomyelitis, synovitis, otitis media, mastoiditis, enteritis, irregular cases of rheumatic fever, meningitis, pleurisy, empyema, and endocarditis. They have also been found as the secondary infection in many diseases, such as in pulmonary tuberculosis, bronchopneumonia, septic diphtheria, diphtheritic scarlatina, measles, smallpox, and others. In the late war streptococci have been reported as the primary or secondary cause of pneumonias and death in a large majority of the cases of respiratory infections among the troops.

In cases of septic thrombus of the lateral sinus following mastoiditis there is almost certainly a streptococcus septicemia. Libman has shown that an examination of the blood may be useful in diagnosis.

In diphtheritic false membranes this micrococcus is very commonly present, and is frequently the source of deeper infection, such as abscesses and septicemia; and in certain cases accompanied by a diphtheritic exudation, in which the Löffler bacillus has not been found by competent bacteriologists, it seems probable that the *Streptococcus pyogenes*, alone or with other pyogenic cocci, is responsible for the local inflammation and its results. These forms of so-called diphtheria, as first pointed out by Prudden, are most commonly associated with scarlatina and measles, erysipelas, and phlegmonous inflammation, or occur in individuals exposed to these or other infectious diseases. So uniformly are long-chained streptococci present in the pseudomembranes of patients sick with scarlet fever, that many investigators have suspected a special variety of them to be the cause of this disease. The same is true for smallpox. Many varieties are regularly found, however, in the throat secretion of healthy individuals (in 100 examinations by us we found long-chained streptococci in 83, and probably could have found them in some of the others by longer search. Their abundance in scarlet fever and smallpox is most probably due to their increase in the injured mucous membrane and entrance into the circulation when the protective properties of the blood have been lowered. Pilot and Davis[1] have recently found that the tonsillar crypts in apparently normal cases frequently contain hemolytic streptococci while the surface of the tonsils show few.

*Septic Sore Throat.*—Streptococcus infection of the throat appears at times as a severe epidemic. Most of these epidemics have been traced to the milk supply. As Smith, Brown,[2] Krumwiede and Valentine[3] and others have shown, that these streptococci probably always come originally from a septic human throat. Streptococci of human origin may invade the milk ducts and multiply in the udder without causing any physical signs of mastitis. The bovine streptococci normally producing mastitis (Mathers[4]) have no relation to septic sore throat.

**Occurrence in Animals.**—Besides streptococci similar to those in man, animals are infected by strains that are negative to Gram and fluidify gelatin. Udder infections of the cow and glandular diseases of the horse are frequently due to these. The streptococcic inflammations in animals are almost as frequent and serious as they are in man.

**Effect on Tumors.**—Fehleisen inoculated cultures, obtained in the first instance from the skin of patients with erysipelas, into patients in the hospital suffering from inoperable malignant growths—lupus, carcinoma, and sarcoma—and he obtained positive results, a typical erysipelatous inflammation having developed around the point of inoculation after a period of incubation of from fifteen to sixty hours. This was attended with chilly sensations and an elevation of temperature. Persons who had recently recovered from an attack of erysipelas frequently proved to be immune. These experiments were undertaken on the ground that malignant tumors had previously been found to improve or entirely disappear in persons who had recovered from accidental erysipelas. This fact was therapeutically applied to the treatment of malignant tumors. Then the mixed toxins of the streptococcus and *B. prodigiosus* were given, and it became apparent that the toxins of the latter organism were much the more important. In some cases of inoperable sarcoma this method met with considerable success (Coley). The injections cause severe reactions.

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[1] Jour. Inf. Dis., 1919, xxiv, 386.

[2] Jour. Med. Res., 1915, xxxi, 455.

[3] Ibid., 1915, xxxiii, 231.

[4] Jour. Infect. Dis., 1916, xix, 222.

**Production of Toxic Substances.**—There is no doubt that the Streptococcus pyogenes causes fever, general symptoms of intoxication, and death by means of toxic substances which it forms in its growth; but we know very little about these substances or how they are produced. The cell substance of streptococci possesses only slight toxicity. Ruediger[1] has shown that a specific streptolysin is formed by Streptococcus pyogenes which stimulates the production of a true antibody. The poisons while partly extracellular are mostly contained in the cell substance. Heat destroys a portion of them. They appear to attack especially the red blood cells, and this hemolytic action seems to be to some degree in proportion to the virulence of the organism.

Clark and Felton[2] have recently described "a filtrable toxic product of the hemolytic streptococcus" produced in growths in rabbits' blood diluted with Locke's solution. When "injected into rabbits in increasing amounts it produced immunity against multiple lethal doses," and also against the organisms themselves.

**Susceptibility to Streptococcus Infection.**—As with the ever-present staphylococci, whose virulence, as we have seen, is usually slight, the streptococci are more likely to invade the tissues, forming abscesses or erysipelatous and phlegmonous inflammation in man when the standard of health is reduced from any cause, and especially when by absorption or retention various toxic organic products are present in the body in excess. It is thus that the liability to these local infections, as complications of operations or sequelæ of various specific infectious diseases, in the victims of chronic alcoholism, and constitutional affections, etc., are tô be explained. It seems established that the absorption of toxic products formed in the alimentary canal as a result of the ingestion of improper food, or in consequence of abnormal fermentative changes in the contents of the intestine, or from constipation predispose to infection.

**Immunity.**—In none of the natural streptococcus inflammations do we notice much apparent tendency to the production of immunizing and curative substances in the blood by a single infection.

Severe general infections usually progress to a fatal termination after a few days, weeks, or months. It is true, however, that cases of erysipelas, cellulitis, and abscess, after periods varying from a few days to months, tend to recover, and to a certain extent, therefore, we may assume that protective agents have been produced. In these cases, however, we know from experience that faulty treatment, by lessening the local or general resistance, would, as a rule, cause the subsiding infection again to progress perhaps even to a more serious extent than the original attack. Koch and Petruschky tried a most interesting experiment. They inoculated cutaneously a man suffering from a malignant tumor with a streptococcus obtained from erysipelas. He developed a moderately severe attack, which lasted about ten days. On its subsidence they reinoculated him; a new attack developed which ran the same course and over the same area. This was repeated ten times with the same results.

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[1] Jour. Am. Med. Assn., 1903, xli, 962; Jour. Infect. Dis., 1906, iii, 663 and 755.

[2] Ibid., 1918, lxxi, 1048.

This experiment proved that in this case, at least, the immunizing substances produced by repeated attacks of erysipelas were insufficient to make the tissues and lymph sufficiently bactericidal to prevent infection.

The severe forms of infection, such as septicemia following injuries, operations, and puerperal infections, show little tendency to be arrested after being well established. Having in mind the above facts, let us consider the results obtained in the experimental immunization of animals.

**Influence of Serum from Immunized Animals upon Streptococcus Infections in Other Animals.**—In the table are given the results following the injection of small amounts of a serum which represents in immunizing value what about one-third of the horses are able to produce when given in gradually increasing doses a living, virulent streptococcus. In the following experiments the serum and culture were injected subcutaneously into rabbits, into some inoculated after mixing and into others separately and on opposite sides of the body.

Showing Strength of Average Grade of Antistreptococcic Serum Given by Selected Horses after Six Months of Injection of Suitable Amounts of Living Streptococci of Pyogenes Type.

| | Weight of rabbit. | Amounts inoculated. Serum | culture. | Results. | Autopsy. |
|---|---|---|---|---|---|
| 1. Inoculated together . . | 1430 | 0.25 c.c. | 0.01 c.c. | Lived | |
| 2. Inoculated together . . | 1350 | 0.125 c.c. | 0.01 c.c. | Lived | |
| 3. On opposite sides . . | 1770 | 0.1 c.c. | 0.01 c.c. | Lived | |
| 4. On opposite sides . . | 1630 | 0.1 c.c. | 0.01 c.c. | Lived | |
| Controls: | | | | | |
| 1. Rabbits injected with culture only. | 1750 | .... | 0.001 c.c. | Died in 4 days | Streptococcic infection. |
| 2. Rabbits injected with culture only. | 1870 | .... | 0.001 c.c. | Died in 24 hrs. | Streptococcic infection. |

The above results have been repeatedly obtained, and are absolutely conclusive that the serum of properly selected animals, which have been repeatedly injected with living streptococci of the pyogenes type in suitable doses, possesses bactericidal properties upon the same type of streptococcus.

**Preparation of the Serum.**—The preparation of antiserums is given in Part III under Applications of Serum Therapy.

**Streptococcus Vaccine.**—The preparation and use of streptococcus vaccines is given in Part III, under Practical Applications of Vaccines.

**Complement Fixation.**—The method and results of this test have been considered in Part I, Chapter XIV.

**The Production of Agglutinins.**—Increasing inoculations of living or dead streptococci into rabbits or other suitable animals will usually produce a considerable amount of agglutinin. As, however, most streptococci are self-agglutinating, tests are read with difficulty. Efforts to overcome this interfering factor have so far met with only partial success.

Recently isolated strains are less self-agglutinative. Until we can study our strains by "the absorption of agglutinin test" (see p. 190) in a satisfactory manner the question of the grouping of streptococci according to definite serologic types will remain unsolved.[1]

**Non-hemolytic Streptococci.**—Non-hemolytic streptococci, most of which produce a green zone (methemoglobin) on blood agar plates, are not of such active virulence as are the hemolytic types. The majority of strains seem to be slowly invasive and may produce chronic inflammation of a low grade. The methemoglobin produced by many of the strains in variable amounts is probably a combination of reduction and oxidation processes (Heubner,[2] Cole[3] and Blake[4]). It has no relation to virulence and very little to carbohydrate reactions. Based on cultural and agglutinative reactions Krumwiede and Valentine[5] showed that strains of this group isolated from a series of human cases are practically heterogeneous

Rosenow and his co-workers[6] claimed that a variety of non-hemolyzing streptococci is the cause of poliomyelitis (see Filtrable Viruses). Mathers[7] and Rosenow[8] have recently claimed that a "peculiar green-producing streptococcus" is the cause of epidemic influenza. (See under Influenza.)

**Bacteriological Diagnosis.**—Streptococci, using the name in a broad sense, can often be demonstrated microscopically by simply making a smear preparation of the suspected material and staining with methylene-blue solution or diluted Ziehl's stain. In order to demonstrate them microscopically in the tissues the sections are best stained by Kühne's methylene-blue method. In all cases, even when the microscopic examination fails, the cocci may be found by the use of culture media, such as broth and plated-blood-agar at 37° C. To obtain them from a case of erysipelas it is best to excise a small piece of skin from the margin of the erysipelatous area in which the cocci are most numerous; this is crushed up and part of it transferred to ascitic or serum bouillon, and part is streaked across freshly solidified agar in a Petri dish on which a drop of sterile rabbit's blood has been placed. Both are kept in the incubator at 37° C.

In septicemia the culture method is always required to demonstrate the presence of streptococci, as the microscopic examination of specimens of blood is not sufficient. For this purpose from 10 to 15 c.c. of the blood should be drawn from the vein of the arm aseptically by means of a hypodermic needle, and to each of three tubes containing 10 c.c. of melted nutrient agar kept at about 43° C., 1 c.c. of blood is added. After thorough mixing, the contents are poured into Petri dishes. The remainder is added to several flasks containing 100 c.c. of nutrient broth, in order to produce a development of the cocci, which are found in small numbers in the blood. Petruschky is of the opinion that the cocci can be best shown in blood by animal inoculation. Having withdrawn from the patient 10 c.c. of blood by means of a hypodermic syringe, under aseptic precautions, he injects a portion of this into the abdominal cavity of a mouse, while the other portion is planted in bouillon. Mice thus inoculated die from septicemia when virulent streptococci are present in only very small numbers in the blood. If a successful inoculation takes place we can, through the absence or presence of the development of capsules, often differentiate between the pneumococcus and the streptococcus. Cultures may fail to do this. The development of a wide, clear zone about the colonies (upon blood agar), without a development of green pigment, indicates that the streptococci belong to the pyogenes type. The absence of a definite zone and the development of a green color indicates that they are pneumococci, or streptococci of the viridans type. The growth in the Hiss inulin serum medium will generally differentiate between these two, as the pneumococci usually coagulate the serum, while the great majority of streptococci do not. The bile test should also be applied (p. 307). Agglutination may also be tried but this would only differentiate certain strains. The morphological and cultural characteristics of the streptococcus give us, unfortunately, no absolute knowledge as to the influence which the protecting serum will have. The actual test is here our only method. The detection of the streptococcus in the blood is in itself an unfavorable prognostic sign.

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[1] Dochez & Coworkers: Jour. Exp. Med., 1919, xxix, 215.

[2] Arch. Exp. Path. u. Pharm., 1913, lxxii, 239.

[3] Jour. Exp. Med., 1914, xx, 363.

[4] Ibid., 1916, xxiv, 315.

[5] Jour. Inf. Dis., 1916, xix, 760.

[6] Ibid., 1918, xxii, pp. 281, 313, 345.

[7] Jour. Am. Med. Assn., 1918.

[8] Ibid., 1919.

The blood cultures in many cases of supposed septicemia give no results, for many of these cases develop their symptoms and even die from the absorption of toxins from the local infection, such as an amputation wound or an infected uterus or peritoneum, and the bacteria never invade the blood. When we get negative results we are, as a rule, utterly unable to test the case with curative serums with any accuracy, for the sepsis may be due to either the streptococcus, colon bacillus, staphylococcus, or some other pathogenic variety of microorganism.

In mixed infections when the streptococci present may not be isolated from original plates, plates made from an eighteen-hour culture in suitable broth may contain many isolated colonies.

Brown, in attempting to standardize methods of differentiation among members of this group, has given very definite directions in regard to the kind and amount of blood and agar to be used for blood-agar plate cultures. It would be well for all laboratories to follow such directions in order better to compare results.

# CHAPTER XVII.

# THE DIPLOCOCCUS OF PNEUMONIA (PNEUMOCOCCUS, STREPTOCOCCUS PNEUMONIÆ, MICROCOCCUS LANCEOLATUS).

## THE DIPLOCOCCUS OF PNEUMONIA.

THE diplococcus of pneumonia was observed in 1880 almost simultaneously by Sternberg and Pasteur in the blood of rabbits inoculated with human saliva. In the next few years Talamon, Friedländer, A. Fränkel, Weichselbaum, and others subjected this microörganism to an extended series of investigations and proved it to be the chief etiological factor in the production of lobar or croupous pneumonia in man. The relationship of this organism to the streptococcus group is spoken of in the preceding chapter.

The outcome of the various investigations proved that the acute lung inflammations, especially when not of the frank lobar pneumonia type, are not excited by a single variety of microörganism, and that the bacteria involved in the production of pneumonias are also met with in inflammations of other tissues.

In any individual pneumonic inflammation it is also found that more than one variety of bacteria may be active, either from the start or as a later addition to the original infection.

Among all the microörganisms active in exciting pneumonia, the diplococcus of pneumonia is by far the most common, being almost always present in primary lobar pneumonia and as frequently as any other germ in acute bronchopneumonia and metastatic forms. Besides the different varieties of pneumococci the following bacteria among others are capable of exciting pneumonia: *Streptococcus pyogenes, Staphylococcus pyogenes, Bacillus pneumoniæ, Bacillus influenzæ, Bacillus pestis, Bacillus diphtheriæ, Bacillus typhi, Bacillus coli*, and the *Bacillus tuberculosis*.

Avery and co-workers[1] found among 529 cases diagnosed clinically and pathologically as acute lobar pneumonia that the following were the etiologic agents demonstrated:

| | |
|---|---|
| Diplococcus pneumoniæ | 454 |
| Streptococcus pyogenes | 7 |
| Bacillus influenzæ | 6 |
| Friedländer's bacillus | 3 |
| Staphylococcus aureus | 3 |
| Streptococcus mucosus | 1 |
| Mixed infection with combinations of Staphylococcus aureus, Friedländer's bacillus, B. influenzæ, St. pyogens and St. viridanes | 6 |
| Undetermined | 49 |
| Total | 529 |

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[1] Avery, Chickering, Cole, Dochez: Monograph of the Rockefeller Institute for Med. Res., 1917, No. 7, New York.

**Morphology.**—Typically, the pneumococcus occurs as spherical or oval cocci, usually united in pairs, but sometimes longer or shorter chains consisting of from three to six or more elements and resembling the streptococcus. The paired cells are usually pointed at one end—hence the name *lanceolatus* or lancet-shaped. When thus united the junction, as a rule, is between the broad ends of the oval, with the pointed ends turned outward; but variation in form and arrangement of the cells is characteristic of this organism, there being great differences according to the source from which it is obtained. As observed in the sputum and blood it is usually in pairs of lancet-shaped elements, which are surrounded by a capsule. (See Fig. 111). When grown in fluid culture media longer or shorter chains are frequently formed similar to those of certain streptococci. On solid medium containing serum or blood the organisms are often elongated into bacillary forms. The pneumococcus is by some classed as a streptococcus. Rosenow claims that a typical pneumococcus may be easily changed into a typical streptococcus. (See preceding chapter.)

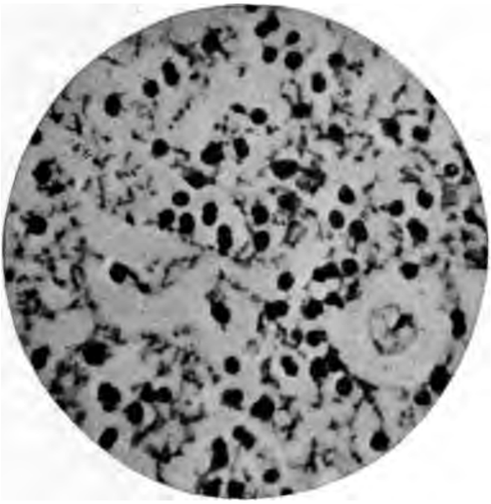

Fig. 111.—Diplococcus of pneumonia from blood, with surrounding capsule stained by method of Hiss.

The capsule is best seen in stained preparations from the blood and exudates of fibrinous pneumonia or from the blood of an inoculated animal, especially the mouse, in which it is commonly, though not always, present. It is seldom seen in preparations from cultures unless special media are employed, except with recently isolated strains of pneumococcus mucosus. The significance of the capsule is not known. It is supposed to serve as a protective agent and to be related to virulence, but supporting evidence is wanting. Flagella are not present.

**Staining.**—It *stains* readily with ordinary anilin colors; it is not decolorized after staining by Gram's method. The capsule may be demonstrated in blood or sputum by the methods given (p. 80).

**Biology.**—It grows equally well with or without oxygen; its parasitic nature is exhibited by the short range of temperature at which it usually grows—viz., from 25° to 42° C.—best at 37° C. In the cultivation of this organism neutral or slightly alkaline media should be employed. The organism when freshly isolated grows feebly on the serum-free culture media ordinarily employed for the cultivation of bacteria—viz., on nutrient agar and gelatin, in bouillon. The best media for its growth are (1) a mixture of one-third human or animal blood serum or ascitic or pleuritic fluid and two-thirds bouillon, (2) nutrient agar streaked with human, horse, or rabbit blood; (3) pneumococcus broth (p. 318).

**Growth on Agar.**—Cultivated on plain nutrient agar, after twenty-four to forty-eight hours at 37° C., the deep colonies are hardly visible to the eye. Under the microscope they appear light yellow or brown in color and finely granular. The surface colonies are larger, equalling in size those of streptococci, but are usually more transparent. If blood serum or ascitic fluid is added to the agar the individual colonies are larger and closer together, and the growth is more distinct in consequence and of a grayish color. The surface colonies are almost circular in shape under a magnification of 60 diameters, finely granular in structure, and may have a somewhat darker, more compact center, surrounded by a paler marginal zone. With high magnification cocci in twos and short rows often distinctly separated are seen at the edges.

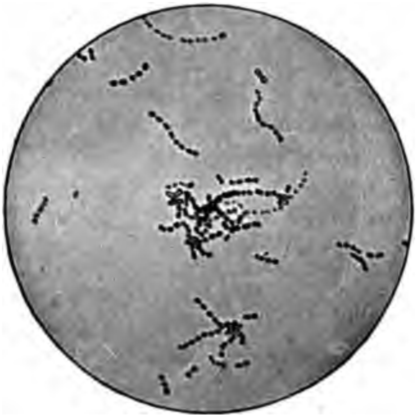

FIG. 112.—Pneumococcus from bouillon culture, resembling streptococcus.

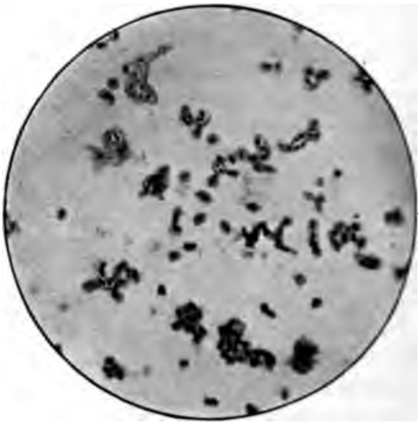

FIG. 113.—Pneumococci stained for capsule by Huntoon's method. (Huntoon.)

**Growth on Blood Agar.**—The colonies on blood agar are greenish with no definite zone of hemolysis. There is a distinct checker-like or ringed edge. Methemoglobin is produced in broth cultures as shown on addition of freshly washed red corpuscle suspensions.

**Growth on Blood Serum.**—The growth on Löffler's blood-serum mixture is very similar to that on agar, but somewhat more vigorous and characteristic, appearing on the surface as a delicate layer of dew-like drops.

**Growth in Bouillon.**—In bouillon, at the end of twelve to twenty-four hours in the incubator, a slight cloudiness of the liquid will be found to have been produced. On microscopic examination cocci can be seen to be arranged in pairs or longer or shorter chains. After one or two transplantations the pneumococci frequently fail to grow.

**Growth in Milk.**—It grows readily in milk causing coagulation with the production of acid, though coagulation is not constant with some forms intermediate between the streptococcus and pneumococcus.

**Growth on Gelatin.**—The growth on gelatin is slow, if there is any development at all, owing to the low temperature—viz., 24° to 27° C.—above which even the most heat-resistant gelatin will melt. The gelatin is not liquefied.

**Special Media.**—When cultures are grown on serum-free media the vitality of some cultures may indeed be indefinitely prolonged; but after transplantation through several generations it is found that the cultures begin to lose in virulence, and that they finally become non-virulent. In order to restore this virulence, or to keep it from becoming attenuated, it is necessary to interrupt the transplantation and pass the organism through the bodies of susceptible animals.

The vitality is prolonged and the virulence less rapidly lost if serum ascitic fluid or blood is present in the medium. Serum or ascitic broth, serum semisolid (stab cultures) or blood-streaked agar are the most satisfactory media for the preservation of cultures.

*Action of Bile and Bile Salts.*—If 0.1 c.c. of rabbits' bile be added to 1 or 2 c.c. of a broth culture of pneumococci the culture becomes clear due to the dissolving of the cocci. A 10 per cent. solution of sodium taurocholate has the same action. Streptococci are not dissolved by bile. Serum and glucose interfere with the reaction. According to Sellards[1] dilute sodium hydroxide helps the solubility in bile of certain strains of pneumococci.

**Hiss Serum-water with and without Inulin.**—These are very useful. The inulin is fermented by typical recently isolated pneumococci with coagulation of the serum, while most streptococci fail to ferment the inulin. This medium is therefore of considerable diagnostic value.

**Calcium Broth with or without Dextrose.**—The addition of a small piece of marble to each tube of broth is the most satisfactory way of preparing it. Marble broth for this purpose was suggested independently by Bolduan and Hiss as very satisfactory for growth.

**Resistance to Light and Drying** (see Media).—On artificial culture media the pneumococci tend to die rapidly. This is partially due to the acid produced by their growth. In sputum they live much longer.

Pneumonic sputum attached in masses to clothes, when dried in the air and exposed to diffuse daylight, retains its virulence, as shown by injection in rabbits, for a period of nineteen to fifty-five days. Exposed to direct sunlight the same material retains its virulence after but a few hours' exposure. This retention of virulence for so long a time under these circumstances is accounted for by the protective influence afforded by the dried mucoid material in which the micrococci were embedded. Guarnieri observed that the blood of inoculated animals, when rapidly dried in a desiccator, retained its virulence for months; and Foá found that fresh rabbit blood, after inoculation and cultivation in the incubator for twenty-four hours, when removed at once to a cool, dark place, retained its virulence for sixty days. There are many conditions, therefore, in which the virulence of the micrococcus is retained for a considerable length of time; the fine spray expelled in coughing and loud speaking that remains suspended in the air soon dries so completely that probably no pneumococci survive after two hours. The action of chemical disinfectants is given in Part III under Disinfection.

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[1] Jour. Am. Med. Assn., 1918, lxxi, 1301.

**Attenuation of Virulence.**—The loss of virulence which occurs when the micrococcus is transplanted through several generations in culture fluid containing no blood has already been referred to. An attenuation of virulence, it has been claimed, takes place also spontaneously in the course of pneumonia. This attenuation is probably only apparent. If a little sputum is taken at different periods in the disease and planted in ascitic bouillon the resultant cultures do not vary greatly in virulence.

**Restoration and Increase of Virulence.**—The simplest and perhaps the most reliable method of restoring lost virulence for any susceptible animal is by passage through the bodies of highly susceptible animals of the same species. Growth in fresh blood also increases it for the homologous animal.

**Maintenance of Virulence.**—This is best done by drying the spleen of an infected animal in a dessicator. The spleen should be removed just before or immediately after death. The virulence is preserved in this way for a month or longer.

**Toxin Production.**—We have little exact knowledge upon the nature of the substances produced by or through the growth of the pneumococci in animal tissues or artificial media. Rosenow showed that the autolysis of virulent pneumococci in NaCl solution brings into the solution a group of substances which inhibits the action of the pneumococci-opsonin. Such extracts are also toxic for animals. Pneumococci dissolved in bile uniformly yield a toxic product. A hemotoxin is present in the bile extracts and can also be extracted by other methods.

**Precipitable Substance.**—This is demonstrable[1] in broth cultures within four to six hours' incubation indicating that the substance may be excreted rather than due to autolysis of the cocci. This substance is demonstrable not only in lung lesion and sputum but also in the blood and thence in the urine. Its concentration in the urine and therefore its successful demonstration is relatively proportionate to the severity of the disease. Aside from its interest from the diagnostic standpoint (see below), it is important as a probable factor in the toxemia of the disease.

**Occurrence in Man during Health.**—It is probable that in crowded communities the pneumococcus is present on the mucous membranes of most persons. We have found it generally present not only in the throats of persons living in New York City,[2] but also in those of persons living on farms and in the Adirondack Mountains. It is commonly present only on the mucous membranes of the bronchi, trachea, pharynx, and nostrils. The healthy lung seems to be generally free from it. The type found is usually one of the less virulent members of Group IV (see below). Carriers of the more virulent types also occur, usually due to contact with a case of pneumonia.

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[1] Dochez and Avery: Jour. Exp. Med., 1917, xxvi, 477.

[2] Report of Respiratory Commission, Department of Health, New York City, Studies on the Pneumococcus, Part I, Reprinted from Jour. Exp. Med., 1905, vii, 401. Studies on the Pneumococcus, Part II, Reprinted from Jour. Infect. Dis., 1906, iii, 774.

**Pathogenicity in Man.**—Pneumococci, characteristic or atypical, are present in fully 95 per cent. of characteristic cases of lobar pneumonia. Usually no other bacteria are obtained from the lungs. Atypical cases usually show the same conditions, but they may be due to streptococci, influenza bacilli, etc. The more recent the infection the greater is the number of bacteria found in the diseased lung area. As the disease progresses these decrease in number until finally at the crisis they disappear from the tissues, though at this time and long after convalescence they may be present in the sputum. In atypical forms of pneumonia they may remain longer in the tissues, and in walking pneumonia they may be absent in the original centers of infection or present only as attenuated varieties, while the surrounding, newly formed foci may contain fully virulent cocci. It has been shown by Netter that more than one-half of the cases of bronchopneumonia, whether primary or secondary to some other disease as measles and diphtheria, both in children and adults, are due to the diplococcus of pneumonia. Others, such as Pearce, have found other microörganisms, especially the streptococci, in the majority of cases. These findings will be considered at the end of the chapter.

The pneumococci are found partly in the alveoli and bronchioles of the inflamed lung and partly in the lymph channels and blood capillaries. Most of the organisms are found free, but a few are found in the leukocytes. Through the lymph channels they find their way to the pleura and to adjacent lymph glands. From the capillaries they find their way to the general blood current, and thus to distant parts of the body. In about 20 per cent. of cases the pneumococci are so abundant that they can be found in cultures made from 5 to 10 c.c. of blood. In a number of instances the fetus has been found infected. The pneumococci are also responsible for:

**Inflammations Complicating Pneumonia.**—In every case of lobar pneumonia and in most cases of bronchopneumonia, pleurisy is developed, which is excited by the same microörganism that was predominant in the pneumonia. With pneumococci the exudate is usually moderate and of a fibrinous character, but may be more abundant and of a serofibrinous or purulent character. When the pleurisy is marked it is more apt to continue after the cessation of the pneumonia. Pleurisy due to pneumococci is more apt to go on to spontaneous recovery than that due to streptococci or staphylococci.

The most frequent pneumococcic infections next to pleurisy, following a pneumonia, are those of the middle ear, pericardium, endocardium, and meninges, and these not infrequently arise together. Pneumococcic inflammations of the heart valves are apt to be followed by extensive necrosis and growth of vegetations. In these cases pneumococci can sometimes be found in the blood for many weeks. Pericarditis due to pneumococci is a frequent complication, but is usually very slightly developed. Meningitis due to pneumococci may be either fibrinous or purulent or both and is apt to be secondary to otitis, mastoiditis, or pneumonia. Arthritis, periarthritis, and osteomyelitis are rarer complications of a pneumococcic pneumonia. Besides moderate parenchymatous inflammation of the kidney, which occurs in most cases of pneumonia, well-marked inflammation may occur in which pneumococci exist in the kidney tissues in large numbers.

The presence of pneumococci in the blood after death has been amply proved by numerous investigations. In many instances, they have been recovered from the blood during life. Lambert, as a rule, found them in all fatal cases twenty-four to forty-eight hours before death. The conveyance of the infective agent by means of the blood and the lymph to all parts of the body explains the multiplicity of the affections complicating a pneumonia, which are caused by this micrococcus; and not only the secondary, but also the primary diseases, as of the brain and meninges, may be explained in the same way.

**Presence in Inflammatory Process Not Secondary to Pneumonia.**—It is now known that the pneumococcus may infect and excite diseases in many tissues of the body independent of any preliminary localization in the lung. As a rule these processes are acute and usually run a shorter and more favorable course than similar inflammations due to the streptococci.

The most frequent primary lesions excited by the pneumococcus after lobar pneumonia, bronchopneumonia, and bronchitis are probably meningitis, otitis media with its complicating mastoiditis, endocarditis, pericarditis, rhinitis, tonsillitis, conjunctivitis, and keratitis; septicemia, arthritis, and osteomyelitis; inflammations of the epididymis, testicles, and Fallopian tubes; peritonitis, etc.

Pneumococcic peritonitis and appendicitis are not so very frequent. The exudate is usually seropurulent.

Conjunctivitis due to pneumococci frequently occurs in epidemic form and is frequently associated with rhinitis.

From statistics collected by Netter the following percentages of diseases were caused by the pneumococcus:

| | | | |
|---|---|---|---|
| Pneumonia | 65.9 | per cent. | in adults. |
| Bronchopneumonia | 15.8 | " | " |
| Meningitis | 13.0 | " | " |
| Empyema | 8.5 | " | " |
| Otitis media | 2.4 | " | " |
| Endocarditis | 1.2 | " | " |

In 46 consecutive pneumococcus infections in children there were:

| | | |
|---|---|---|
| Otitis media | 29 | cases. |
| Bronchopneumonia | 12 | " |
| Meningitis | 2 | " |
| Pneumonia | 1 | case. |
| Pleurisy | 1 | " |
| Pericarditis | 1 | " |

The pneumococcus and streptococcus are the two most frequent organisms found in otitis media. The cases due to the pneumococcus are apt to run the shorter course, but have a tendency to spread to the meninges and cause a meningitis. The pneumococci may also find their way into the blood current. This usually follows after sinus thrombosis.

In bronchitis the pneumococcus is frequently met alone or in combination with the streptococcus, the influenza bacillus, or other bacteria.

In certain epidemics pneumococcic bronchitis and pneumonia simulate influenza very closely and cannot be differentiated except by bacteriological examinations.

Primary pneumococcic pleurisy is frequent in children: it is very often purulent, but may be serous or serofibrinous. Its prognosis is better than that in cases due to other organisms. Frequently we have streptococci and staphylococci associated with the pneumococci.

**Pathogenesis in Lower Animals.**—Most strains of the *Micrococcus lanceolatus* are moderately pathogenic for numerous animals; mice and rabbits are the most susceptible, indeed some strains are intensely virulent for these animals, while guinea-pigs and rats are much less susceptible. Pigeons and chickens are refractory. In mice and rabbits the subcutaneous injection of small or moderate quantities of pneumonic sputum in the early stages of the disease, or of a twenty-four-hour ascitic broth culture from such sputum, or of a pure, virulent ascitic broth culture of the micrococcus, usually results in the death of these animals in from twenty-four to forty-eight hours. The course of the disease produced and the postmortem appearances indicate that it is a form of septicemia—what is known as sputum septicemia. After injection there is loss of appetite and great debility, and the animal usually dies some time during the second day after inoculation. The postmortem examination shows a local reaction, which may be of a serous, fibrinous, hemorrhagic, necrotic, or purulent character; or there may be combinations of all of these conditions. The blood of inoculated animals immediately after death often contains the micrococci in very large numbers. For microscopic examination they may be obtained from the blood, and usually from pleural and peritoneal exudates when these are present.

True localized pneumonia does not usually result from subcutaneous injections into susceptible animals, but injections made through the thoracic walls into the substance of the lung may induce a typical fibrinous pneumonia. This was first demonstrated by Talamon, who injected the fibrinous exudate of croupous pneumonia, obtained after death or drawn during life from the hepatized portions of the lung, into the lungs of rabbits. Wadsworth showed that by injecting virulent pneumococci into the lungs of rabbits which had been immunized, a typical lobar pneumonia was excited, the bactericidal property of the blood being sufficient to prevent the general invasion of the bacteria. Pneumonia may be produced in dogs, and in rabbits less easily, by intratracheal injections.

**Varieties of the Pneumococcus.**—As among all other microörganisms minutely studied, different strains of pneumococci show quite a wide range of variation in morphology and virulence. Some of the variations are so marked and so constant that they make it necessary to recognize several distinct varieties of the pneumococcus, and to class as pneumococci certain varieties which have before this been classed as streptococci—*e. g.*, the so-called Streptococcus mucosus capsulatus (Streptococcus mucosus Schottmüller)—when first isolated from pneumonic exudate or elsewhere, and planted on artificial culture media containing serum, grows as a rounded coccus with a small dense distinct capsule, principally in short or medium chains; it produces a large amount of mucus-like zoöglea, forming very large spreading colonies; it promptly coagulates fluid-serum media containing inulin. It is also very virulent for mice, but only moderately virulent for rabbits. After a number of culture generations on ordinary nutrient agar it apparently loses most of these characteristics. It then grows in small colonies principally as naked diplococci which may be elongated and pointed, produces no zoöglea, and loses most of its virulence for mice and rabbits. It still coagulates inulin-serum media, and when transferred to serum media regains its former morphological characteristics. For these reasons we consider this organism a distinct variety of the pneumococcus. This variety of pneumococcus has been isolated by us from the lungs after death following lobar pneumonia, out of twenty consecutive autopsies, as the only organism present twice, and with another variety of pneumococcus once. Together with other varieties it was isolated from four out of twenty specimens of pneumonic sputum, and five times from sixty specimens of normal throat secretion. In 1905 Park and Williams showed that this variety should be placed with the pneumococci under the common name *pneumococcus mucosus.* (Type III of Avery.)

**Agglutination Reactions and Determination of Types.** — That agglutinins are produced in animals by the injection of pneumococci was shown by Neufeld, Clairmont, and others. It has since been shown that this test may be used as a means of diagnosis. Neufeld,[1] Collins, Cole[2] and Dochez,[3] and others have shown that certain pneumococci may be grouped according to serum reactions. According to Cole and Dochez, the groups based upon their agglutination reactions are as follows: Group I and II, typical pneumococci; Group III, pneumococcus mucosus; Group IV, heterogeneous strains. Most strains in the last group seem independent as far as serum reactions are concerned; it is really not a group but rather an assembling together of isolated strains, and Avery[4] has shown that occasionally pneumococci occur which are related to Group II in that the serum of a type organism protects animals against infection by these subtypes, at least to a very considerable degree, but the reverse, that a serum produced by one of them protects against the others or the standard Type II, has not been demonstrated. More than one subgroup[1] evidently exists. Variations in the agglutinative characteristisc of pneumococci can be induced but not a change from one group to another. Passage through a susceptible animal causes a return of its type reaction.

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[1] Neufeld and Händel: Pneumococcus, Handbuch der pathogenen Microörganismen. Kolle and Wassermann, 2 Aufl., Bd. iv.

[2] Arch. Int. Med., 1914, xiv, 56 (general discussion with bibliography).

[3] Jour. Exp. Med., 1912, xvi, 663.

[4] Ibid., 1916, xxiv, 7 (see also ibid., xxiv, 25).

The following table by Cole gives the types he found in pneumonia in a series of cases occurring in New York City, and the relative mortality of the cases according to the infecting type:

| Infecting type. | Cases. | Strain, per cent. | Deaths. | Mortality, per cent. |
|---|---|---|---|---|
| I | 34 | 47 | 8 | 24 |
| II | 13 | 18 | 8 | 61 |
| III | 10 | 14 | 6 | 60 |
| IV | 15 | 21 | 1 | 7 |
| Total | 72 | 100 | 23 | 32 |

In different localities, and at different times in the same locality, the type percentage varies greatly and the mortality also varies somewhat. Reports of the many pneumonias recovering during the late war show a distinct difference in percentage of types. Lister, in South Africa, found a type distinct from Types I, II or III to be the prevailing type.

Wollstein and Benson[2] found that Group IV occurred more frequently in the pneumonias of children and that the mortality due to this group was high (40 per cent.). Only a limited number of observations are available on the groups occurring in meningitis. Valentine gives the following series: I, 2 cases; II, 7 cases; III, 2 cases; IV, 2 cases. Total, 13.

Pneumococci taken from normal throats or from slight inflammation fall usually into Group IV among the heterogeneous strains. The strains belonging to Group I are widely scattered, being found not only throughout the United States but also in Europe.

**Bacteriological Diagnosis.**—Direct smears may be prepared and stained with the Gram and with the capsule stain. The microscopic findings may be verified by preparing "pour or streak" blood plates, isolation of pure cultures and determination of bile solubility. Isolation may also be made by intraperitoneal mouse inoculation (see p. 148). As a septicemic infection usually develops, the presence of the typical cocci is easily demonstrable in the peritoneal exudate and in the heart's blood, from which cultures can then be isolated.

**Rapid Methods for the Determination of Type.**—*Sputum.*—The sample should come from the deeper air passages and as free as possible from saliva. Three methods are available: (*a*) "demonstration of precipitable substance" in sputum, (*b*) "cultural method," (*c*) mouse inoculation method." Before attempting any of these tests there should be available antisera for Types I, II and III which have been tested for presence of cross-reactions and minimal dilutions determined for specific testing. The less cross-action there is the better are they suited for this purpose as lower dilutions may be used, which leads to quicker reactions.

[1] Stillman: Jour. Exp. Med., 1919, xxix, 251.

[2] Am. Jour. Dis. Child., 1916, xii, 254.

Precipitable substance in the sputum is most easily determined by the coagulation method of Krumwiede and Valentine. The technic of the test is as follows:

"From 3 to 10 c.c. of the sputum, depending on the amount available, is poured from the sputum container into a test-tube. This is placed in boiling water for several minutes or longer until a more or less firm coagulum results, which will occur if the specimen is a suitable one. The coagulum is then broken up with a heavy platinum wire or glass rod, and saline is added. Just enough saline should be added so that, on subsequent centrifuging, there will be sufficient fluid to carry out the test. If too much is added, the resulting antigen may be too dilute. In some instances little or no saline is necessary, as sufficient fluid separates from the coagulum.

After the addition of the saline, the tube is again placed in boiling water for a few minutes to extract the soluble antigen from the coagulum, the tube being shaken several times during the heating. The broken clot is then thrown down by centrifuge, and the clear supernatant fluid used for the test. To hasten the appearance of the reaction and to obtain a reaction even should the antigen be dilute, we float the antigen over the "type" serums, using the latter undiluted. Two-tenths c.c. of the three "type" serums are placed in narrow test-tubes, and the antigen added from a capillary tube with a rubber teat. If the tubes containing the serum are tilted and the antigen dropped slowly on the side of the tube just above the serum, no difficulty will be encountered in obtaining sharp layers, as the undiluted serum is sufficiently higher in its specific gravity. The tubes are then placed in the water-bath at from 50° to 55° C. and observed after several minutes.

If a fixed type was present in the sputum, and should the sputum have been rich in antigen, a definite contact ring is seen in the tube containing the homologous serum. With sputums less rich in antigen, the ring may develop more slowly, and it will be less marked. Some experience is necessary in detecting the less marked contact rings and in differentiating them from an apparent ring which may be confusing, if one of the serums is darker in color, giving thus a sharper contrast with the supernatant antigen. The true ring is more or less opaque, and this quality can be seen by tilting the tubes and looking at the area of contact against a dark background, for example, the lower edge of a dark shade raised to just above the level of the eyes. The advantage of the ring test is that a ring may be evident, whereas definite clouding or visible precipitate may appear only after longer incubation, or may be so slight even after an hour's incubation as to leave one in doubt. It is well to shake the tubes after twenty minutes, as many of the specimens will show definite clouding or precipitate either at once or on further incubation, thus checking the ring reading. A positive reaction usually appears within one-half hour and the majority are evident in less than ten minutes."

The rapid cultural method of Avery[1] is carried out as follows:

A small portion of sputum about the size of a bean is washed, through three or four changes of sterile saline, ground up in a mortar and emulsified with 0.5 to 1 c.c. of broth and the whole inoculated into a tube of 4 c.c. of the following medium. The medium is 100 c.c. of infusion broth (+ 0.3 to 0.5 of phenolphthalein) to which is added 5 c.c. of a sterile 20 per cent. solution of glucose and 5 c.c. of sterile defibrinated rabbits' blood. (We use 5 c.c. of citrated horse blood.) After inoculation, shake and incubate in the water-bath for five hours. Centrifuge at low speed to throw down the red corpuscles and collect supernatant culture. Make smear, if apparently pure, set up agglutination test mixing 0.2 c.c. of the culture with 0.2 c.c. of each of the type sera if necessary diluting the latter (see above) and incubate and observe up to one hour. If smear shows presence of other organisms add 1 c.c. of sterile ox-bile to culture, incubate for twenty minutes at 37° C. to cause solution of pneumococci, then centrifuge at high speed till clear and then mix 0.2 c.c. of this with each of the type sera, incubate and observe for one hour.

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[1] Jour. Am. Med. Assn., 1918, lxx, 17.

The mouse inoculation method is as follows:

A bean-sized mass of the sputum, preferably washed and emulsified as above, is injected intraperitoneally into a white mouse. The pneumococci multiply rapidly in the mouse peritoneum. After five to eight hours the mouse will show evidence of illness especially if one of the fixed types was present in the sputum. The mouse is then killed the peritoneal cavity opened and the exudate washed into a sterile petri dish with 4 to 5 c.c. of saline, using a pipette. A smear taken from the exudate will indicate the presence or absence of other organisms. The peritoneal washings are centrifuged at low speed to throw down the cells and fibrin. The supernatant fluid is transferred to another tube and centrifuged at high speed till the fluid is clear. This is carefully removed. One may then proceed, utilizing this clear fluid for the precipitin test[1] or one may resuspend the sedimented cocci in saline (density of broth culture) and utilize this for the agglutination. The precipitin method is especially valuable where other bacteria are present. The bacillus of Friedländer and the influenza bacillus are the types most likely to "come through" the mouse.

The above methods are reliable in direct proportion to the care taken in obtaining the sample of sputum. The success of the coagulation method depends on the content of coagulable substance which will be greater the more nearly the sputum is really a pulmonary exudate. Even when satisfactory coagulation occurs, a negative reaction does not exclude an infection by a fixed type, as very little antigen may be present. The method will give from 70 per cent. to 90 per cent. correct tests in fixed type infections, thus allowing prompt serum administration if indicated. The Avery method is limited by the same factors, source and purity, and a negative finding may mean overgrowth by a Group 4 variety from the mouth. The majority of specimens which are satisfactory for the Avery method also give a positive reaction with the coagulation test. The mouse method is less subject to error as even the presence of contaminating Group IV varieties may not vitiate the test as the more virulent fixed types will come through in preponderance sufficient for test purposes, even though originally present in small numbers. As Type III occurs with some frequency in normal mouths, its development in the culture or mouse method is not necessarily an indication that it is the infecting agent. For statistical purposes the above methods should be checked by isolating pure cultures from the sputum or from the mouse heart for verification tests with the immune sera.

*Urine* may be utilized for the determination of the infecting type. This method is of less value, however, because the demonstrable amounts of precipitable substance appears in only about 65 per cent. (usually the more severe cases), and as a rule the sputum examination is earlier applicable. The test is as follows:

Clear urine by centrifuge and set precipitin reaction as above. To concentrate the antigen, acidify 25 c.c. of urine by the addition of a few drops of acetic acid and boil down to 5 c.c., filter, and add to 8 to 10 volumes of 95 per cent. alcohol, collect the precipitate that forms (by centrifuge) dissolve in 2 c.c. of saline, clear by centrifuge and use clear fluid for precipitin test.

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[1] **Blake: Jour. Exp. Med., 1917, xxvi, 67.**

Cerebrospinal fluid and other exudates will frequently give a precipitin reaction, utilizing the clear fluid obtained by centrifuge. The sediment may be utilized for mouse inoculation.

**Immunity.**—Following an attack of pneumonia some immunity is established, but this lasts only a short time. After successive injections of gradually increasing doses of virulent pneumococci into certain animals (horse, sheep, goat, rabbit), a serum of protective and some curative power in experimental animals is obtained. The mode of action of this serum is still the subject of study. According to Wright, Neufeld, and others, its activity is due primarily to the opsonins. The mechanism of the crises in pneumonia is not understood, but the presence of increased opsonins and other protective substances at this time suggests that it is a phenomenon dependent upon the increase of these substances and associated cellular activity.

*Serums and Vaccines* are given in Part III under Applied Therapy.

**Chemotherapy.**—Morgenroth and Levy[1] showed that ethylhydrocuprein (optochin), a derivative of hydroquinone, was of value in the treatment of experimental pneumococcus infections. This substance has an almost specific action on pneumococci and *in vitro* its greater action on the pneumococcus group serves to separate this group from the related cocci.

It acts even in dilutions as great as 1 in 1,000,000. It has a protective and curative action in guinea-pigs and mice infected with pneumococci. Small doses of optochin increase considerably the protective power of the type homologous antipneumococcus serum in experimental animals (Moore).

The pneumococcidal action of the serum after administration of the drug was studied in rabbits by Moore[2] and in patients suffering from acute lobar pneumonia by Moore and Chesney.[3] These observers state that when patients receive by mouth 0.024 to 0.026 gram of optochin hydrochloride every twenty-four hours per kilogram of body weight, the serum acquires a pneumococcidal action and that the drug is apparently helpful in the treatment of lobar pneumonia due to pneumococci.

The use of the drug in humans may give rise to amblyopia or amaurosis, which is generally transitory if the drug be discontinued when these symptoms appear. Moore and Chesney have collected from the literature 786 cases of lobar pneumonia treated with the drug, among which the mortality was 12.84 per cent.; the eye symptoms referred to above occurred in 4.4 per cent. of the cases and in one of these this disturbance was more or less permanent.

Tagendreich and Russo have shown that pneumococci subjected to sublethal concentrations of optochin in the test-tube can be rendered "fast," or resistant to the drug within a few days, and Morgenroth has shown that the same phenomenon can be observed to take place *in vivo* (mice) inefficiently treated. Moore and Chesney recovered a "fast" strain of pneumococcus from one of their fatal cases treated with optochin.

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[1] Berl. klin. Wchnschr., 1911, xlvii, 1560, 1979.

[2] Jour. Exp. Med., 1915, xxxi, 389, 557.

[3] Arch. Int. Med., 1917, xix, 611.

# CHAPTER XVIII.

# MENINGOCOCCUS OR MICROCOCCUS (INTRACELLULARIS) MENINGITIDIS, AND THE RELATION OF IT AND OF OTHER BACTERIA TO MENINGITIS.

WHILE, as has already been stated, the pneumococcus is a cause of isolated cases of meningitis the meningococcus is the most frequent cause of purulent meningitis either sporadic or endemic. In 1887 Weichselbaum[1] discovered a micrococcus in the exudate of cerebrospinal meningitis in 6 cases, 2 of which were not complicated by pneumonia. He obtained it in pure cultures, studied its characteristics and showed that this organism was clearly distinguishable from the pneumococcus and especially by its usual presence in the interior of pus cells, on which account he called it Diplococcus intracellularis meningitidis. In 1895 Jaeger and Scheurer drew especial attention to the etiological relationship of the organism to the epidemic form of cerebrospinal meningitis. They also believed it to be very probable that in most cases of primary meningitis it is from the mucous membrane of the nasal cavities and the sinuses opening out from them that both the diplococcus of pneumonia and the micrococcus intracellularis find their way through the blood or perhaps directly through the lymph channels to the meninges. Both we know to be very frequently present in the nasal cavities. The nasal secretion of carriers is more dangerous to the community than that of infected persons because carriers are not recognized as such usually and mingle freely with the general public. The prevalence of epidemics in winter and spring, a time favorable to influenza and pneumonia, also suggests the respiratory tract as the place of the infection and where an increase in virulence takes place. We do not as yet know why meningitis follows in some persons and not in others after infection of the mucous membranes.

**Morphology.**—This organism occurs as biscuit-shaped micrococci, usually united in pairs, but also in groups of four and in small masses, sometimes solitary, and small degenerated forms are found. It has no well-defined capsule. Cultures resemble strongly those of gonococci. In the sediment from spinal fluids (see Fig. 114) meningococci are both intra- and extracellular. When they are intracellular the prognosis is better, and frequently in cases where they are extracellular at first they later become intracellular.

**Staining.**—They stain with all the ordinary aniline dyes and are readily decolorized by Gram's solution. A smear from a culture shows characteristic irregularity in staining, some of the cocci taking the counterstain poorly and some staining deeply. The positive cocci described by Jaeger and others were probably contaminating organisms.

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[1] Fortschr. d. Med., 1887, p. 573.

**Cultivation.**—They grow between 25° and 38° C., best at about 35° C. They are easily isolated on 2 per cent. glucose ascitic agar neutral to phenolphthalein. We have found that the so-called "vitamin" agar with the addition of blood (p. 123) is the best medium for direct isolation. They are kept well on semisolid media. The liver medium recommended by Dopter (p. 129) is an excellent one for stock transplants. The reaction of the media is very important. A culture can rarely be isolated on plain nutrient agar.

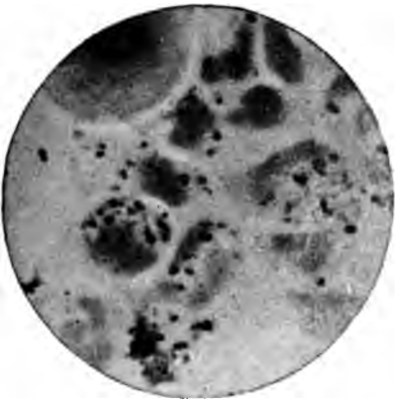

Fig. 114.—Diplococcus intracellularis meningitidis in pus cells. × 1100 diameters.

Different strains of meningococci vary somewhat in the ease with which they may be cultivated, virulence for animals, as well as agglutinability, degree of digestibility in leukocytes, and power of resistance to immune serum.

After having been isolated for some time, a tolerably good growth develops at the end of forty-eight hours in the incubator. On semitransparent media (glucose-ascitic agar) the colonies may be seen as a flat layer, each about ⅛ inch in diameter, grayish white in color, finely granular, rather viscid, and non-confluent unless very close together. On vitamin-blood-agar the colonies may grow to a small pinhead size in eighteen hours. They have a characteristic ground glass appearance. In forty-eight hours the great majority of the strains develop in the center of the colonies, the crystals described by Elser and Huntoon.[1] Gonococcus colonies may also develop similar crystals but not in such great abundance. From the spinal fluid in acute cases, where the organisms are apt to be more abundant, a great many minute colonies may develop instead of a few larger ones. On agar plates the deep-lying colonies are almost invisible to the naked eye; somewhat magnified they appear finely granular, with a dentated border. On the surface they are larger, appearing as pale disks, almost transparent at the edges, but more compact toward the centers, which are yellowish-gray in color. On blood agar or serum agar the growth is much more luxuriant than on plain agar and larger than the gonococcus. Cultivated in artificial media, while it often lives for weeks, it may die within four days, and requires, therefore, to be transplanted to fresh material at short intervals.

**Resistance.**—It is readily killed by heat, disinfectants, sunlight, and drying. A few cocci may remain alive for one to three days in the dried state. To maintain cultures it is necessary to make transfers frequently. Dried rapidly under freezing temperature and kept frozen they may remain alive for years (Elser and Huntoon).

**Agglutination.**—Different strains vary very much in their agglutinability. Even different antigens from one and the same strain may show a similar degree of variation. The agglutination test is utilized as a routine in the identification of cultures from other sources than the cerebrospinal fluid. The slide agglutination method, using a 1 to 10 or greater dilution of immune horse serum (the dilution depending on preliminary tests with known strains) and a similar dilution of a normal horse serum as a control, is a great convenience in examining the colonies from plates, when searching for carriers.[1] The drops after drying are used for control by staining with the Gram stain. The macroscopic tube agglutination may also be employed, using agar cultures isolated from the colonies. The latter method is less expedient and in practical work seems to have no advantage over the slide method except possibly for verification purposes. In these tests a serum of a horse immunized with many varities of meningococci is utilized. It is evident that such a serum, because of group reactions, may give a positive reaction with Gram-negative cocci which are not meningococci. With our present lack of knowledge as to the limits of the meningococcus group, they must be considered meningococci or at least potential agents for the production of meningitis. We are unable therefore to identify all meningococci with surety. If, however, a strain can be identified as belonging to one of the four dominant groups (see below), we can definitely say it is a true meningococcus. As varieties, not belonging to such groups, are encountered in meningitis, the agglutination reactions are of no value in indicating the virulence of a culture.

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[1] Jour. Med. Res., 1909, xx, 377.

**Groups or Varieties of Meningococci.**—Elser and Huntoon,[2] Wollstein[3] and others found differences in the serum reactions of strains. The results indicated a tendency to grouping but were apparently not sufficiently constant or clean-cut to warrant a definite separation. Dopter found that certain strains differed rather sharply both agglutinatively and on absorption from the general type and suggested the term "parameningococcus" for such aberrant varieties. Gordon[4] has extended the use of the method of agglutinin absorption and believes that four distinct types (groups?) exist as well as an apparently heterogeneous group. He has found that the majority of the strains coming from meningitis cases fall into four main groups. He numbers his types I, II, III and IV. Types I and III and Types II and IV are related. Tulloch[5] has repeated Gordon's findings but his results indicate the existence of subvarieties of Type II. We have been working on the absorptive capacity of meningococci and the results thus far obtained indicate the existence of the Gordon groups but we do not believe they are as sharply defined or as homogeneous as the results of Gordon and others would seem to indicate. One point should be emphasized, viz., that the direct agglutination with specific type sera is only of relative value in determining the group to which a culture belongs. Even marked or complete cross-agglutination may be noted with two strains having distinctly different agglutinin absorptive capacities. Even immune sera from the rabbit, will show these marked cross-reactions.

The question of determining the existence of types of meningococci and their relative prevalence is of the greatest importance in the production of therapeutic serums. How far cross-agglutination is associated with cross-protection is an important factor concerning which we have no information.

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[1] **Krumwiede**: Jour. Am. Med. Assn., 1917, lxix, 358.

[2] **Elser and Huntoon**: Jour. Med. Res., 1909, xx, 377. Griffith: Jour. of Hyg., 1915–17, xv, 446; Griffith: Jour. of Hyg., 1918, xvii, 124. Nicolle: Ann. de l'Inst. Past., 1918, p. 154.

[3] **Wollstein**: Jour. Exp. Med., 1914, xx, 201.

[4] Jour. Roy. Army Med. Corps, 1915, xxv, 411, also Jour. Hyg., 1918, xvii, 290.

[5] Ibid., 1912, xxix, No. 1. See also Dopter et Pauron: Comptes Rend. de la Soc. de Biol., 1914, lxxvii, 157.

**Pathogenesis.**—This organism has a low grade and very variable pathogenicity for laboratory animals. Following a suitable intraperitoneal injection the temperature of the guinea-pig falls, the hair stands out, the abdomen becomes distended and the muscles rigid. The animal hunches up in a corner and seems very sick. Not infrequently there is prolapse of the rectum. If the dose is fatal death usually occurs in from ten to forty-eight hours. At autopsy there is fluid exudate into the abdomen and sometimes into the pleural cavity, congestion or hemorrhage of the adrenals, hemorrhages into the mesentery, central tendon of the diaphragm and into the whole peritoneum. Frequently it is possible to recover the meningococcus from the heart's blood when live culture has been used. The organisms do not have to multiply to produce death and the autolysate or killed culture is just as fatal as live culture. Death is probably due to a bacterial poison freed by the disintegration of the meningococci. Very young cultures are apt to produce a septicemia. Rabbits injected either subcutaneously or intravenously lose weight rapidly.

**Pathogenicity for Man.**—The most marked lesions occur at the base of the brain. The cord is always affected. This is not true to the same extent in other bacterial infections. In some epidemics the course of the disease is very rapid. The mortality without serum treatment varies between 50 and 80 per cent.

**Presence of Meningococci in the Nasal Cavity of the Sick and Those in Contact with Them.**—In 6 of his 6 cases Weichselbaum succeeded in obtaining diplococci from the nasal secretion. In 1901 Albrecht and Ghon demonstrated them in healthy individuals. Scheurer, in 18 cases, found the diplococci in the nasal secretions during life. In 50 healthy individuals examined they were found in the nasal secretions of only 2, 1 being a man suffering at the time from a severe cold. This man, it is interesting to note, had been employed in a room which had just previously been occupied by a patient with cerebrospinal meningitis.

During the recent war much work was done on the detection of meningococcus carriers and with the better culture media and technic of collecting material (use of West tube) large numbers of carriers were detected. Whether or not all of the strains isolated were virulent remains to be determined.

**Presence in other Localities.**—In addition to the situation already noted, the meningococcus is frequently found in the blood in the early days of the disease. Elser found it in blood cultures in 10 out of 40 cases. The meningococci have also been found in the herpes and in the urine, a fact to be considered in ordering the hygiene of the sick room.

**Complicating Infections.**—Occasionally we find secondary to the cerebrospinal meningitis, and due to the *Micrococcus pneumonia*, cystitis, conjunctivitis, inflammation of the middle ear, arthritis, endocarditis, etc. The most frequent and serious complication of meningococcic meningitis is pneumonia probably due in many cases to the pneumococcus.

Details in regard to the administration of serum and of vaccine are given in Part III under Applied Therapy.

**Laboratory Diagnosis.**—*Spinal Fluid.*—The fluid should be collected in a sterile container. It may be clear, cloudy, or bloody. If it is clear it may be a normal fluid or a fluid from a case of poliomyelitis or tuberculous meningitis.

If it is cloudy it may be due to the meningococcus, streptococcus, pneumococcus, pneumococcus mucosus, influenza bacillus, or other rarer organisms. The blood in a bloody fluid may be due to a previous hemorrhage or to the accidental piercing of a vein. The two conditions, may be differentiated by centrifuging the fluid. If the supernatant fluid is yellow or reddish the hemorrhage is old. Clear fluid indicates a recent hemorrhage. It is unfortunate to have much blood in the fluid for it obscures the microscopic picture and then unless the culture is positive it is difficult to make even a tentative diagnosis. Clear fluids should always be centrifuged preferably for one hour at high speed. Cloudy fluids showing no organisms should also be centrifuged. The sediment should be used to make cultures and smears. The smears are examined: (1) for pus cells and (2) for tubercle bacilli if the fluid is clear or for other organisms if it is cloudy. The sediment from clear fluids should always be stained with the tubercle stain and that from cloudy fluids with Gram's stain. Once in a long while a cloudy fluid will be found to be tuberculous or a clear fluid to be due to some pyogenic organism but these occurrences are so rare as to be practically negligible. Gram's stain differentiates influenza bacillus, and meningococcus from the streptococcus, pneumococcus, and pneumococcus mucosus. The finding of Gram-negative cocci either intra- or extracellular is presumptive evidence of meningococcic meningitis, but it is always well to follow it up with a culture. In order that cultures may grow, fluids should be examined at the earliest moment possible, for meningococci in fluids over twelve hours old are frequently autolyzed so that they will not grow.

The following table gives the main differential points in making diagnosis from spinal fluids:

| | Pressure. | Amount, c.c. | Appearance. | Cytology. | Bact. | Albumin. | Globulin. | Animal inoc. |
|---|---|---|---|---|---|---|---|---|
| Normal | Normal | 5–10 | Clear | Very few cells | Sterile | ± | — | Negative |
| Meningismus | Increased | 10–100 | Clear | Very few cells | Sterile | ± | — | Negative |
| Infantile paralysis | Increased | 20–100 | Clear; sometimes slight fibrin web | Early polynucleosis; later lymphocytosis up to 98 per cent; endothelial cells | Sterile | +–++ | +++ | Negative or pneumonia |
| Tuberculous meningitis | Increased | 30–120 | Clear; fibrin web | Lymphocytosis up to 98 per cent. | Tubercle bacilli | ++–+++ | ++–+++ | Tuberculosis in 4 weeks. |
| Epidemic cerebrospinal meningitis | Increased | 5–120 | Cloudy | Polynucleosis up to 98 per cent. | Meningococcus | ++–++++ | ++–++++ | |
| Meningitis due to other organisms | Increased | 20–100 | Cloudy | Polynucleosis up to 98 per cent. | Infecting organisms | ++–++++ | ++–++++ | |

**Other Gram-negative Cocci Resembling Meningococci.—Micrococcus Pharyngis (Siccus) (von Lingelsheim), Chromogenic Gram-negative Cocci, Micrococcus Catarrhalis.**—These may be differentiated by cultural characteristics.

*Differential Diagnosis with the Sugars.*—As a rule the portion of the body from which the organisms are obtained reveals their identity. When this is insufficient careful cultural and serological tests are required. McNeil has obtained a specific complement-fixation reaction (see p. 258).

The following table gives the chief sugar reactions of this group of microörganisms. (Elser and Huntoon.)

| Organism. | Glucose. | Maltose. | Mannitose. | Levulose. | Saccharose. |
|---|---|---|---|---|---|
| Gonococcus | + | − | − | − | − |
| Meningococcus | + | + | − | − | − |
| M. Catarrhalis | − | − | − | − | − |
| M. Flavus | + | + | + | + | − |
| M. Pharyngis | + | + | + | + | + |

**Other Organisms Exciting Meningitis.**—1. The tubercle bacillus. This is the most frequent cause of meningitis due to an organism other than the meningococcus.

2. The Pneumococcus. This diplococcus is one of the most frequent exciters of meningitis, both as a primary and a secondary infection.

3. The Streptococcus pyogenes and the staphylococcus. Meningitis due to these organisms is almost always secondary to some other infection, such as otitis, tonsillitis, erysipelas, endocarditis, suppurating wound of scalp and skull, etc.

4. The Bacillus influenzæ. Numerous reports have been published of the presence of influenza bacilli in the meningeal exudate. Those that are reliable state in almost every instance that the meningitis is secondary to infection of the lungs, bronchi, and the nasal cavities with their accessory sinuses.

5. The colon bacillus, the typhoid bacillus, that of bubonic plague and of glanders, all may cause a complicating purulent meningitis.

6. In isolated cases of meningitis complicating otitis media and other infections, other bacteria, such as the Micrococcus tetragenus, the Bacillus pyocyaneus, the gonococcus, etc., may be found.

Meningitis due to other organisms than the meningococcus is almost invariably fatal.

## MICROCOCCUS CATARRHALIS (B. PFEIFFER).

Micrococci somewhat resembling meningococci are found in the mucous membranes of the respiratory tract. At times they excite catarrhal inflammation of the mucous membranes and pneumonia. These cocci are at present included under the designation of Micrococcus catarrhalis.

**Microscopic Appearance.**—They usually occur in pairs, sometimes in fours; never in chains. The cocci are coffee-bean in shape, slightly larger than the gonococcus, and are negative to Gram's stain.

The micrococci are not motile and produce no spores.

**Cultivation.**—They grow between 20° and 40° C., best at 37° C. and less rapidly at somewhat lower temperatures, developing on ordinary nutrient agar, as grayish white or yellowish white, circular colonies of the size of meningococci. The borders of the colonies are irregular and abrupt as though gouged out. The colonies have a mortar-like consistency. On serum-agar media the growth is more luxuriant. Gelatin is not liquefied. Bouillon is clouded, often with the development of a pellicle. Milk is not coagulated, but dextrose serum media may be. Gas is not produced.

**Location of Organisms.**—In the secretion of normal mucous membranes they are occasionally present. In certain diseased conditions of the mucous membranes they may be abundant.

**Pathogenic Effects in Animals.**—For white mice, guinea-pigs, and rabbits, some cultures are as pathogenic as meningococci, while others are less so.

**Differential Points Distinguishing them from the Meningococci.**—These organisms have undoubtedly been at times confused. Some assert that the meningococci grow only above 25° C. Many cord cultures of meningococci grow below this point. Some assert that the meningococci will not grow on 5 per cent. glycerin agar. Many undoubted cultures do. Careful agglutinin-absorption tests are of great differential value, but can only be carried out safely by one accustomed to them. The meningococci tested by us have removed all the agglutinins acting upon meningococci from a specific meningococcus serum while the allied organisms have removed only about 60 per cent. of them. The probability is that the organisms described by different writers as Micrococcus catarrhalis were not at all the same variety, and some of them were meningococci.

**Vaccine Therapy.**—This is given in Part III.

## CHAPTER XIX.

## THE GONOCOCCUS OR MICROCOCCUS GONORRHEÆ. MICROCOCCUS MELITENSIS.

THE period at which gonorrhea began to afflict man is unknown. The earliest records make mention of it. Wherever civilized man has penetrated, gonorrhea is prevalent among the people. Except for a period after the fifteenth century it was generally recognized as a communicable disease and laws were made to control its spread. The differentiation between the lighter forms of gonorrhea and some other inflammations of the mucous membranes was, however, almost impossible until the discovery of the specific microörganism by Neisser in 1879.

The organism was first observed in gonorrheal discharges and described by him under the name of "gonococcus;" but though several attempted to discover a medium upon which it might be cultivated, it was reserved for Bumm, in 1885, to obtain it in pure culture upon coagulated human blood serum, and then after cutivating it for many generations to prove its infective virulence by inoculation into man. The researches of Neisser and Bumm established beyond doubt that this organism is the specific cause of gonorrhea in man. Gonorrhea is in almost all cases among adults transmitted through sexual intercourse. Gonorrheal ophthalmia is a frequent accidental infection at birth, and vaginitis in the young child is frequently produced by the carelessness of the nurse or mother carrying infection.

**Microscopic Appearance.**—Micrococci, occurring mostly in the form of diplococci. The bodies of the diplococci are broadened and, as shown in stained preparations, have an unstained division or interspace between two flattened surfaces facing one another, which give them their characteristic "coffee-bean" or "kidney" shape. The older cocci lengthen, then become constricted in their middle portion, and finally divide, making new pairs (Fig. 115). The diameter of an associated pair of cells varies according to their stage of development from $0.8\mu$ to $1.6\mu$ in the long diameter—average about $1.25\mu$—by $0.6\mu$ to $1\mu$ in the cross diameter.

**Extracellular and Intracellular Position of Gonococci.**—In gonorrhea, during the earliest stages before the discharge becomes purulent, the gonococci are found mostly free in the serum or plastered upon the epithelial cells, but later almost entirely in small, irregular groups in or upon the pus cells and epithelial cells, and always extranuclear. With the disappearance of the pus formation more free gonococci appear. Discharge expressed from the urethra usually contains more free organisms than the natural flow. Gonococci sometimes appear irregular or granular, the so-called involution forms. These are found particularly in older cultures and in chronic urethritis of long standing. Single pus cells sometimes contain as many as one hundred gonococci and seem to be almost bursting and yet show but slight signs of injury. These diplococci are also found in or upon desquamated epithelial cells. There is still discussion as to whether the gonococci actively invade the pus cells or only are taken up by them. There is no evidence that the gonococci are destroyed by the pus cells (Fig. 116). In gonorrhea of the conjunctiva they are contained in the epithelial cells, sometimes in large numbers. They form dense groups which contain forms similar to those seen in older cultures, showing metachromatic granules in round, swollen, pale blue bodies. These groups finally present an appearance somewhat like the cell inclusions found in "trachoma" (p. 465).

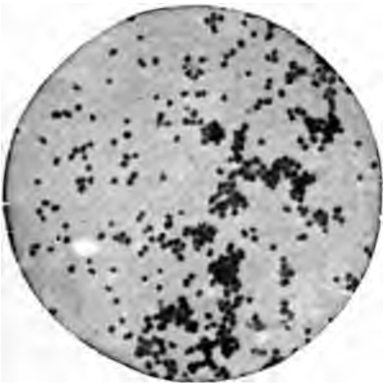

Fig. 115.—Smear from pure culture of gonococcus on agar. × 1100 diameters. (Heiman.)

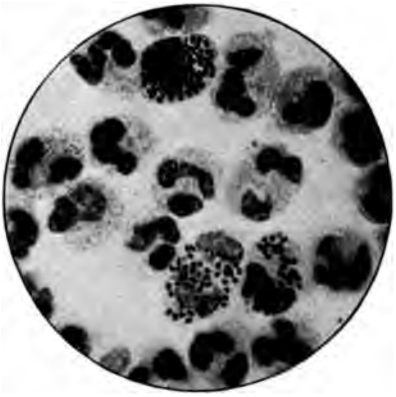

Fig. 116.—Gonococcus within pus cells, fuchsin stain. ×1000 diameters. (Fränkel and Pfeiffer.)

**Staining.**—The gonococcus *stains* readily with the basic anilin colors (p. 76). Löffler's solution of methylene blue is one of the best staining agents for demonstrating its presence in pus, for, while staining the gonococci deeply, it leaves the cell protoplasm but faintly stained. Fuchsin is apt to overstain the cell substance. Beautiful double-stained preparations may be made from gonorrheal pus by treating cover-glass smears with methylene blue and eosin. Numerous methods for double staining have been employed, with the object of making a few gonococci more conspicuous. None of them has any specific characteristics such as the Gram stain. It is now established that gonococci from fresh cultures and from recent gonorrheal infections are, when properly treated by Gram's method, quickly and surely robbed of their color and take on the contrast stains. The removal of the stain from gonococci in old flakes and threads from chronic cases is not so certain. This difference is mostly due to the fact that equally uniform specimens cannot be prepared. The decolorized gonococci are stained by dipping the films for a few seconds into a 1 to 10 dilution of carbol-fuchsin or a solution of Bismarck brown. This staining should be for as short a time as suffices to stain the decolorized organisms. This method of staining cannot be depended upon alone absolutely to distinguish the gonococcus from all other diplococci found in the urethra and vulvovaginal tract, for, especially in the female, other diplococci are occasionally found which are also not stained by Gram's method. It serves, however, to distinguish this micrococcus from the common pyogenic cocci, which retain their color when treated in the same way, and in the male urethra it is practically certain, as few organisms have been found in that location which in morphology and staining are identical with the gonococcus. It is certainly the most distinctive characteristic of the staining properties of the gonococcus, and it is a test that should never be neglected in differentiating this organism from others which are morphologically similar.

**Biology.**—Grows best at blood temperature; the limits being roughly 25° and 40° C. It is a facultative anaërobe. It is not motile and produces no spores.

**Culture Media.**—The gonococcus requires for its best growth the addition to nutrient agar of a small percentage of blood serum or some equivalent. The media which have proved of value may be found in the chapter on Media. We have found that they grow very well on heated blood mixtures such as vitamin blood agar. Ruediger[1] has also gotten good results with similar media with partial exclusion of air, thus confirming our observations on the facultative anaërobic nature of this organism.

After continued cultivation gonococcus cultures frequently grow on media containing no serum. Occasional strains grow on ordinary glycerin or glucose nutrient agar and even on plain nutrient agar from the start, but their growth is more capricious in such media.

**Viability.**—Cultures usually die in forty-eight to seventy-two hours when kept at room temperature. In the ice-box they may live for several weeks. They frequently live for one week in the thermostat at 36° C. on and in semisolid media.

**Appearance of Colonies.**—A delicate growth is characteristic At the end of twenty-four hours there will have developed translucent, very finely granular colonies, with scalloped margin. The margin is sometimes scarcely to be differentiated from the culture medium. In color they are grayish white, with a tinge of yellow. The texture is finely granular at the periphery, presenting yellowish punctated spots of higher refraction in and around the center (Fig. 117).

**Surface Streak Culture.**—Translucent grayish-white growth, with rather thick edges.

**Resistance.**—The gonococcus has but little resistant power toward outside influences. It is killed by weak disinfecting solutions and by desiccation in thin layers. In comparatively thick layers, however, as when gonorrheal pus is smeared on linen, it has lived for forty-nine days, and dried on glass for twenty-nine days (Heiman). It is killed at a temperature of 45° C. in six hours and of 60° in about thirty minutes.

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[1] Jour. Infect. Dis., 1919, xxiv, 376.

**Occurrence of Gonococci in Nature.**—Outside of the human body or material carried from it gonococci have not been found.

**Pathogenesis.**—Non-transmissible as gonorrhea to all test animals. Both the living and dead gonococci contain toxic substances. Injected in considerable amounts into rabbits, they cause infiltration and often necrosis. Applied to the urethral mucous membrane of human beings there is produced an inflammation of short duration. In gonorrhea the secretion is believed to be due to intracellular toxins. Repeated injections give only slight immunity. The filtrate of recent gonococcus cultures contains little toxin.

The etiological relation of the gonococcus to human gonorrhea has been demonstrated beyond question by the infection of a number of healthy men with the disease by the inoculations of pure cultures of the microörganism.

**Disease Conditions Excited by Gonococci.**—Affections due to this organism are usually restricted to the mucous membranes of the urethra, prostate, neck of bladder, cervix uteri, vagina, and conjunctiva. The conjunctival, vaginal, and rectal mucous membranes are much more sensitive in early childhood than in later life. The usual course of the inflammation is as follows: The gonococci first increase upon the mucous membranes which show congestion, infiltration with serous exudate and accumulation of leukocytes. The cocci then penetrate the epithelial layer down to the submucous connective tissue. Recovery or a prolonged chronic inflammation may then persist. The original infection of the urethra or vagina and cervix may remain localized or spread to adjacent parts or through blood and lymph be carried to all parts of the body. Gonococci thus cause some cases of endometritis, metritis; salpingitis, oöphoritis, peritonitis, prostitis, cystitis, epididymitis, and arthritis. Abscesses of considerable size, periostitis, and otitis are occasionally due to the gonococcus.

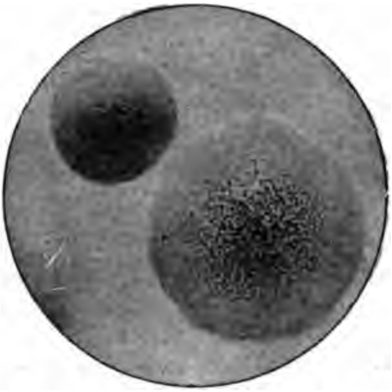
Fig. 117.—Colonies of gonococci on pleuritic fluid agar. (Heimann.)

*Gonorrheal Ophthalmia.*—We have corroborated the statement of Stephenson and others that the gonococcus, though a frequent cause of ophthalmia neonatorum is not the only cause, in fact, in only about two-thirds of these cases is the gonococcus the cause of the inflammation.[1]

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[1] **Williams and Rosenberg:** Arch. of Ophthal., 1916, xlv, 109.

*Endocarditis and Septicemia.*—Cases of gonococcus endocarditis and septicemia are not infrequent. Gonococcus septicemia may occur in connection with other localizations or alone. Nearly every year one or two of these cases are met with in every general hospital. In a considerable number of cases where gonococci are obtained from the blood the patients recover. The fever is sometimes typhoid-like in character.

**Complications.**—General infections with gonococci are often followed or accompanied by neuralgic affections, muscle atrophies, and neuritis. Urticaria occasionally occurs.

**Immunity.**—Immunity in man after recovery from infection seems to be only slight in amount and for a short period if present at all. It is known that the urethra in man or cervix uteri in woman may contain gonococci which lie dormant and may be innocuous in that person for years, but which may at any time excite an acute gonorrhea in another individual or, under stimulating conditions, in the one carrying the infection. Animals, may, however, be immunized, and their blood is both bactericidal and slightly antitoxic.

**Use of Serum and Vaccine.**—The use of vaccine and of serum are given in Part III under Practical Applications of Vaccines and Serums.

**Complement Fixation.**—By the method of McNeil (p. 258) very good results have been obtained in the deep-seated chronic infections, but the reverse is true in the superficial acute cases. The method is used regularly in the New York City Health Department.

**Agglutination.**—Torrey has shown that gonococci resemble pneumococci in that there are a number of different strains which have different specific and but few common agglutinins. The agglutination test is of no practical value in diagnosis.

**Duration of Infections and of Contagious Period.**—There is no limit to the time during which a man or woman may remain infected with gonococci and infect others. We have had one man under observation in which twenty years had elapsed since exposure to infection, and yet the gonococci were still abundant. It is now well established that most of the inflammations of the female genital tract are due to gonococci and that many of such infections are produced in innocent women by their husbands who are suffering from latent gonorrhea.

**Bacteriological Diagnosis of Gonorrhea.**—In view of the fact that occasional non-gonorrheal forms of urethritis exist, and also that micrococci morphologically similar to Neisser's diplococcus are at times found in the normal vulvovaginal tract of adults, it becomes a matter of importance to be able to detect gonococci when present, and to differentiate these from the non-specific organisms. Besides this, the gonococci which occur in old cultures and in chronic urethritis of long standing sometimes take on a very diversified appearance. From a medicolegal and social standpoint, therefore, the differential diagnosis of the gonococcus has in certain cases a very practical significance.

There are three methods of differential diagnosis now available—

the microscopic, the cultural, and complement fixation. The method employed in the last test is given on p. 258. Animal inoculations are of no value, as animals are not susceptible, and, of course, human inoculations are usually impossible. In the microscopic diagnosis it should be borne in mind that after the acute serous stage has passed, the specific gonococci in *carefully made* preparations are always found largely within the pus cells. Diplococci morphologically similar to gonococci occurring in other portions of the field and outside of the pus cells should not be considered specific by this test only. It should also be remembered that the gonococci are decolorized by Gram's method, while other similar micrococci which occur in the urethra are, as a rule at least, not so decolorized. Organisms having these characteristics can for all practical purposes be considered as certainly gonococci if obtained from the urethra. From the vulvovaginal tract the certainty is not so great, since other diplococci are found in pus from this area more frequently than from the urethra which stain as gonococci; here cultures should also be made.

In *chronic urethritis* Heiman allows the patient to void his urine either immediately into two sterilized centrifuge tubes or first into two sterile bottles. The first tube will contain threads of the anterior urethra; the second tube will be likely to contain secretion from the posterior urethra and from the prostate gland if, while urinating, the patient's prostate be pressed upon with the finger. Tubes containing such urine are placed in the centrifuge and whirled for three minutes at twelve hundred or more revolutions per minute, at which speed the threads are thrown down. The centrifuged sediment will be found to contain most of the bacteria present, epithelial cells, and, at times, spermatozoa.

When the examinations are negative and it is important to be certain either massage or injections of a solution of silver nitrate may be employed. The latter by causing a temporary irritation with increase of secretion will almost surely cause a discharge of gonococci if any infection is present. It has been recommended that inoculations of vaccine be given to provoke an increase of the number of gonococci in chronic cases. Pearson[1] states that this treatment gave positive results in 96 out of 100 consecutive cases.

In acute cases where the pus is abundant the specimen for examination may be collected, by passing a sterilized platinum-wire loop as far up into the urethra as possible and withdrawing some of the secretion.

**In vulvovaginitis** the procedure should be as follows:

For obtaining the vaginal material the labia are held well apart by an attendant wearing sterile rubber gloves. A sterile slender cotton swab is used which passes easily into the vagina without touching any external part but entrance. (If pus from the cervix is desired a speculum should be used.) The swab is rubbed gently about vaginal wall, then withdrawn and rolled (not rubbed) quickly over a slide (slide sterilized and held face down if culture is to be made). In making this smear care is used not to pass the swab over the same surface twice. In this way a beautifully spread film is made. The swab is then returned to its holder (and covered and numbered if culture is to be made). The air-dried slide is rewrapped in its filter paper, numbered and sent to laboratory where it is stained by Gram's method (p. 79).

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[1] Jour. of Urology, 1918, ii, 455.

*The technic for making culture is as follows:* A small amount of rich sterile ascitic fluid is added to the tubes containing the swab. (If patient is at a distance from laboratory the ascitic fluid is sent in a separate tube, and is inoculated from swab just after smear is made; then swab is withdrawn from ascitic fluid tube, placed in its own tube, and both tubes are sent immediately to the laboratory.) After being stirred up in this fluid the swab is withdrawn and smeared in strokes radiating from the centre over ascitic agar (1–4) plate containing 2 per cent. glucose. Other plates of the same medium are stroked with platinum loopfuls of the ascitic fluid emulsion. From two to four plates are made and placed in thermostat at 36° C. After twenty-four hours the plates are examined and if gonococcus-like colonies are seen they are fished. Then a smear is made from the whole of one of the most characteristic streaks stained by Gram's method and examined for gonococcus-like organisms.

*Grouping Cases.*—From the microscopic examination of well-made and well-stained smears (stained by Gram's method), and, when necessary, from cultures and from clinical appearance as well, the cases are divided into four groups, as follows:

1. *Positive cases, i. e.*, those showing leukocytes filled with morphologically typical gonococci in smear or showing typical cultures, or showing both.
2. *Suspicious cases, i. e.*, those showing in smears any suspicious intracellular diplococci and 50 per cent. or more of polymorphonuclear leukocytes.
3. *Observation cases, i. e.*, those showing in smears 50 per cent. or over of polymorphonuclear leukocytes, but no suspicious intracellular diplococci; or those having the clinical symptoms of discharge and inflammation and showing less than 50 per cent. of polymorphonuclear leukocytes.
4. *Negative cases. i. e.*, those showing in smears less than 50 per cent. polymorphonuclears, and no suspicious intracellular diplococci, and no clinical evidence of the disease.

*Isolation of Groups.*—Each group is kept isolated.

*Later Smears.*—From the first three groups smears are made once a week until a negative smear is obtained. From negative groups smears are made if any suspicious symptoms appear.

*Negative Diagnosis from Later Smears.*—Three successive well-made and well-stained negative smears from first three groups, at intervals of not longer than three days, are considered a negative diagnosis.

*Later Cultures.*—From cases where morphologically typical gonococci persist in smear, cultures may be made and gonococcus-like organisms isolated and studied to find out if they are true gonococci.

**Bacteria Resembling Gonococci.**—A few micrococci which resemble gonococci in form and staining have been described. These assume importance largely because they may be confused with the gonococcus. They occur occasionally on the conjunctival and vaginal mucous membranes. One of these microörganisms, the *Micrococcus catarrhalis* (see p. 326), has an importance of its own. Others are probably unimportant. When absolute certainty is demanded cultural and serological tests must be applied. Differential diagnosis from meningococci is given on p. 327.

## MALTA FEVER.

**The Micrococcus Melitensis.**—This microörganism was first discovered in the spleen in a case of Malta fever by Bruce in Malta in 1887. The disease is chiefly confined to the shores of the Mediterranean, but cases of it have been observed elsewhere. Infected goat herds have been found in Texas.

**Clinical Symptoms.**—Prodromal symptoms follow an incubation period of five to fourteen days. Headache, sleeplessness, loss of appetite, or vomiting accompany a high fever. The fever lasts for weeks, with intermissions and remissions. A fever period of one to three weeks may occur from time to time during a period of many months. The spleen and liver are enlarged. The mortality is slight.

**Autopsy.**—The spleen is large and very soft. The liver is also large and congested. Both organs show parenchymatous degeneration.

**Distribution of Micrococci.**—These are most abundant in the blood and at the height of the fever and are present in organs and in the urine from the second day to the end of the disease.

**Morphology and Biology.**—Very small rounded or slightly oval organisms, about 0.3$\mu$ in their greatest diameter. They are usually single or in pairs. In old cultures involution, almost bacillary, forms occur. They are not motile and are Gram-negative.

**Cultivation.**—At 37° C. they grow rather feebly on nutrient agar and in broth. The colonies are not usually visible until the third day. They appear as small round disks, slightly raised, with a yellowish tint in the center. The broth is slightly clouded after four to six days. The culture remains alive for several weeks or months. In gelatin the growth is very slow. Gelatin is not liquefied.

**Pathogenesis in Animals.**—Monkeys are susceptible. They pass through the disease much like man. They can be infected by subcutaneous or oral inoculation. Guinea-pigs and rabbits are less easily infected. Infected goats pass the organisms in feces, urine, and milk. The milk is believed to be the chief source of infection. By safeguarding the milk the disease has been largely eliminated. Contact infection cannot, however, be completely excluded. Horses and cows are also susceptible.

**Methods of Diagnosis.**—The diagnosis of Malta fever can frequently only be made with the help of bacteriological examination. Blood cultures during the febrile period or cultures of the urine are usually employed. An agglutination reaction with the patient's serum, in dilutions of 1 to 1000 or higher is diagnostic.

Animals injected with the coccus produce a serum agglutinating in high dilutions. This can be used to identify suspected cultures.

## MICROCOCCUS ZYMOGENS.

MacCallum and Hastings[1] observed this micrococcus in a case of acute endocarditis. It has since been found in a few other pathological processes. It occurs in pairs and short chains. It grows well on agar, ferments lactose and glucose, and slowly liquefies gelatin.

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[1] Jour. Exp. Med., 1899, iv, 521.

# CHAPTER XX.

# THE BACILLUS AND THE BACTERIOLOGY OF DIPHTHERIA.

The lesions of diphtheria are caused by toxemia. The concentrated poison at the seat of the exudate causes intense local inflammation, while in the more severe cases the absorbed poison diffused throughout the body causes widespread cellular injury, giving rise to definite lesions of the cells of muscle, nerve, and other tissues.

**Historical Notes.**—This specific contagious disease can be traced back under various names to almost the Homeric period of Grecian history. From time to time during the following centuries we hear of epidemics both in Italy and in other portions of the civilized world which indicate that the disease never absolutely ceased.

In 1771 Bard, an American, advanced strong reasons for believing that membranous croup and pharyngeal diphtheria were different manifestations of the same disease process.

In 1821 Bretonneau published his first essay on diphtheria in Paris and gave to the disease its present name. His observations were so extensive and so correct that little advance in knowledge took place until the causal relations of the diphtheria bacilli and their associated microörganisms to the disease began to be recognized.

**Evidence of Causal Relationship.**—As early as 1840 observers began to notice microörganisms in the pseudomembranes. Gradually the observations became more exact. The most importance was attributed to micrococci. In the year 1883, however, bacilli which were very peculiar and striking in appearance were shown by Klebs to be of constant occurrence in the pseudomembranes from the throats of those dying of true epidemic diphtheria. He described the peculiar staining of the organisms. One year later Löffler separated these bacilli from the other bacteria and grew them in pure culture. They have therefore been called Klebs-Löffler bacilli. When Löffler inoculated the bacilli upon the abraded mucous membrane of susceptible animals more or less characteristic pseudomembranes were produced, and frequently death or paralysis followed with characteristic lesions. These animal experiments have been fortified by a number of accidental human inoculations in laboratories with pure cultures of bacilli with subsequent development of diphtheria.

**The Diphtheria Bacillus.**—This bacillus is one of the most interesting of bacteria. Grown in the animal body or in suitable culture fluid, it produces a powerful toxin. Its morphology and staining are peculiar. Outside of the body it grows best on serum media.

**Morphology.**—When cover-glass preparations made from the exudate or from the cultures grown on blood serum, are examined, the diphtheria bacilli are found to possess the following morphological characteristics: The diameter of the bacilli varies from 0.3μ to 0.8μ and the length from 1μ to 6μ. They occur singly and in pairs (see Figs. 118 to 125) and very infrequently in chains of three or four. The rods are straight or slightly curved, and usually are not uniformly cylindrical throughout their entire length, but are swollen at the end, or pointed at the ends and swollen in the middle portion. The average length of the bacilli in pure cultures from different sources frequently varies greatly, and even from the same culture individual bacilli differ much in their size and shape. This is especially true when the bacilli are grown in association with other bacteria. The two bacilli of a pair may lie with their long diameter in the same axis or at an obtuse or an acute angle. The bacilli possess no spores, but have in them highly refractive bodies, some of which are the starting-point for new bacilli. There are no flagella. For mode of division see p. 37.

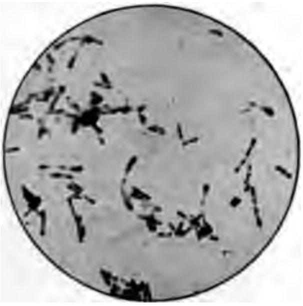

FIG. 118.—One of the very characteristic forms of diphtheria bacilli from blood-serum cultures, showing clubbed ends and irregular stain. × 1100 diameters. Stain, methylene blue.

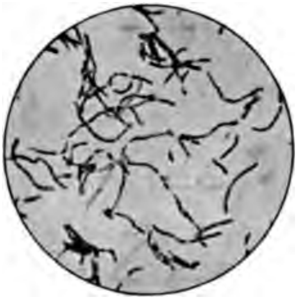

FIG. 119.—Extremely long form of diphtheria bacillus. This culture has grown on artificial media for over twenty years and produces great amounts of toxin. × 1100 diameters.

**Staining.**—Diphtheria bacilli *stain* readily with ordinary anilin dyes, and retain fairly well their color after staining by Gram's method. With Löffler's alkaline solution of methylene blue, and to a less extent with Roux's and dilute Ziehl's solution (page 78), the bacilli from blood-serum cultures especially, and from other media less constantly, stain in an irregular and extremely characteristic way. (See Fig. 118.) The bacilli do not stain uniformly. In many cultures round or oval bodies, situated at the ends or in the central portions, stain much more intensely than the rest of the bacillus, usually showing metachromatism (metachromatic granules. See page 37 and Plate III.) Sometimes these highly stained bodies are thicker than the rest of the bacillus; again, they are thinner and surrounded by a more slightly stained portion Other bacilli have barred staining. The bacilli stain in this peculiar manner at a certain period of their growth, so that only a portion of the organisms taken from a culture at any one time will show the characteristic staining. The young cultures have the most regular forms, an eighteen-hour growth showing more clubbed forms than at twelve

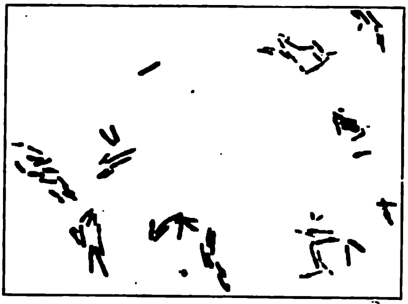

FIG. 120.—Diphtheria bacilli characteristic in shapes, but showing even staining. × 1000 diameters. Stain, methylene blue.

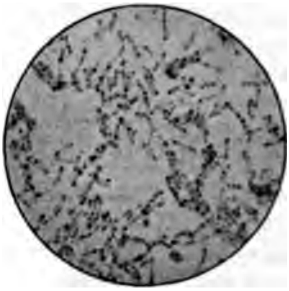

FIG. 121.—Non-virulent diphtheria bacilli, showing stain with Neisser's solutions. This appearance was formerly supposed to be characteristic of virulent bacilli. Bodies of bacilli in smear, yellowish brown; granules, dark blue.

FIG. 122.—B. diphtheria agar culture. Bacilli small and uniform in shape. × 1000 diameters.

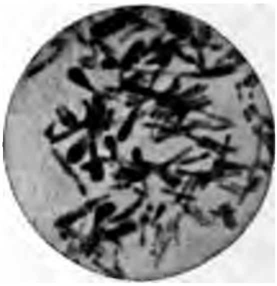

FIG. 123.—B. diphtheriæ. Forty-eight hours' agar culture. Thick, Indian-clubbed rods and moderate number of segments. One year on artificial culture media. × 1410 diameters.

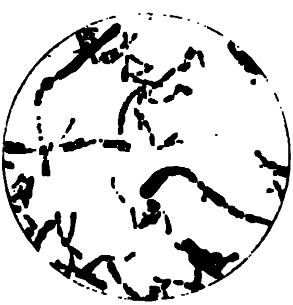

FIG. 124.—B. diphtheriæ. Forty-eight hours' agar culture. Many segments; long, Indian-clubbed ends. One year on artificial media. × 1410 diameters.

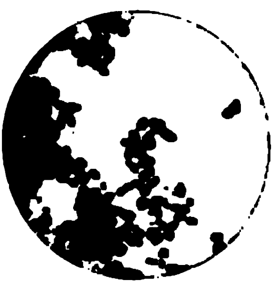

FIG. 125.—B. diphtheriæ. Twenty-four hours' agar culture. Coccus forms. Segmented granular forms on Löffler's serum. Only variety found; in cases of diphtheria at Children's Home. × 1410 diameters.

hours. After twenty-four hours the bacilli do not stain quite as well. In still older cultures it is often difficult to stain the bacilli, and the staining, when it does occur, is frequently not at all characteristic. The same round or oval bodies which take the methylene blue more intensely than the remainder of the bacillus are brought out still more distinctly by the Neisser stain (page 79).

The Neisser stain has been advocated in order to separate the virulent from the non-virulent bacilli, without the delay of inoculating animals; but in our hands, with a very large experience, neither the Neisser stain nor other stains, such as the modifications of the Roux stain, have given much more information as to the virulence of the bacilli than the usual methylene-blue solution of Löffler. A few strains of virulent bacilli fail to show a marked characteristic stain, and quite a few pseudodiphtheria bacilli show the dark bodies. There are also in many throats bacilli which seem to have all the staining and cultural characteristics of the virulent bacilli, and yet have no relation to the disease diphtheria, that is, they produce no diphtheria toxin. As will be stated more fully later, nothing but animal inoculations with the suspected bacilli together with control injections of diphtheria antitoxin will separate harmless bacilli from those capable of producing diphtheria.

**The Morphology of the Diphtheria Bacillus on Serum-free Media.**—This varies considerably with different culture media employed. On glycerin agar or simple nutrient agar there are two distinct types. One grows as smaller and, as a rule, more regular forms than when grown on serum culture media (Fig. 121). The other type shows many thick, Indian-club forms with a moderate number of segments (Figs. 122–124). Short, spindle-, lancet-, or club-shaped forms, staining uniformly, are all observed. The bacilli which have developed in the pseudomembranes or exudate in cases of diphtheria resemble in shape young bacilli grown on agar.

**Biology.**—The Klebs-Löffler bacillus is non-motile and non-liquefying. It is *aërobic* and facultative *anaërobic*. It grows most readily in the presence of oxygen. It does not form spores. It begins to develop but grows slowly at a temperature of 20° C. or even less. It attains its maximum development at 37° C. In old cultures in fluid media Williams has observed fusion of one bacillus with another. The fused forms live the longest (p. 98).

**Growth on Culture Media.**—**Blood Serum.**—Blood serum, especially coagulated in the form of Löffler's mixture, is the most favorable medium for the growth of the diphtheria bacillus, and is used particularly for diagnostic purposes in examining cultures from the throats of persons suspected of having diphtheria. For its preparation see p. 122. If we examine the growth of diphtheria bacilli in pure culture on blood serum we shall find at the end of from eight to twelve hours small colonies of bacilli, which appear as pearl-gray, whitish-gray or, more rarely, yellowish-gray, slightly raised points. The colonies when separated from each other may increase in forty-eight hours so that the diameter may be one-eighth of an inch. The colonies lying together become confluent and fuse into one mass when the serum is moist. The diphtheria colonies after a growth of twelve hours become larger than those of the streptococci but remain smaller than those of the staphylococci.

**Growth on Agar.**—On 1 per cent. slightly alkaline, nutrient or glycerin-agar the growth of the diphtheria bacillus is less certain and luxuriant than upon blood serum; but the appearance of the colonies when examined under a low-power lens, though very variable, is often far more characteristic (Fig. 126). For this reason nutrient agar in Petri dishes is used to obtain diphtheria bacilli in pure culture. Certain strains of the diphtheria bacillus after having been transplanted for several generations on serum culture media, grow well, or fairly well, on suitable nutrient agar, but when fresh from pseudomembranes they grow on this medium with great difficulty, and the colonies develop so slowly as to be covered up by the more luxuriant growth of other bacteria when present; or they may fail to develop at all.

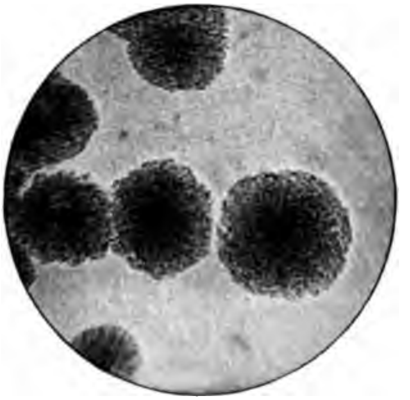

FIG. 126.—Colonies of diphtheria bacilli. × 200 diameters.

If the colonies develop deep in the substance of the agar they are usually round or oval, and, as a rule, present no extensions; but if near the surface, commonly from one, but sometimes from both sides, they spread out an apron-like extension, which exceeds in surface area the rest of the colony. When colonies develop entirely on the surface they are more or less coarsely granular, and usually have a dark center and vary markedly in their thickness. The colonies from some are almost translucent; from others are thick and almost as luxuriant as the staphylococcus. The edges are sometimes jagged, and frequently shade off into a delicate lace-like fringe; at other times the margins are more even and the colonies are nearly circular.

Peculiarities in the growth of the diphtheria bacillus upon agar are of practical importance. If a large number of the bacilli from a recent culture are implanted upon a properly prepared agar plate a certain and fairly vigorous growth will always take place. If, however, the agar is inoculated with an exudate from the throat, which contains but a few bacilli, no growth whatever may occur, while the tubes of coagulated blood serum inoculated with the same exudate contain the bacilli abundantly. Because of the uncertainty, therefore, of obtaining a growth by the inoculation of agar with bacilli unaccustomed to this medium, agar is not a reliable medium for use in primary cultures for diagnostic purposes. A mixture composed of 2 parts of a 1.5 per cent. nutrient agar and 1 part of sterile ascitic fluid makes a medium upon which the bacillus grows much more luxuriantly, but not so characteristically.

**Growth in Bouillon.**—The diphtheria bacilli from about one-half the cultures grow readily in broth slightly alkaline to litmus; the other strains grow feebly. The characteristic growth in neutral bouillon is one showing fine grains. These deposit along the sides and bottom of the tube, leaving the broth nearly clear. A few cultures in neutral bouillon and many in alkaline bouillon produce for twenty-four or forty-eight hours a more or less diffuse cloudiness, and frequently a film forms over the surface of the broth. On shaking the tube this film breaks up and slowly sinks to the bottom. This film is apt to develop during the growth of cultures which have long been cultivated in bouillon, and, indeed, after a time the entire development may appear on the surface in the form of a friable pellicle. The diphtheria bacillus in its growth, causes a fermentation of meat sugars and glucose, and thus, if these are present, changes the reaction of the bouillon, rendering it distinctly less alkaline within forty-eight hours, and then, after a variable time, when all the fermentable sugars have been decomposed, more alkaline again through the progressing fermentation of other substances. Among the products formed by its growth is the diphtheria toxin.

**Growth in Ascitic or Serum Bouillon.**—All varieties of diphtheria bacilli grow well in this medium, even when first removed from the throat. They almost always form a slight pellicle at the end of twenty-four which increases by the end of forty-eight hours. This culture medium is, as pointed out by Williams, of the greatest value in attempts to get pure cultures of the diphtheria bacillus from solidified serum cultures containing few bacilli among many other bacteria. Plate cultures are made from the pellicle. The fluid is prepared by adding to the nutrient bouillon 25 per cent. ascitic fluid or blood serum.

**Growth in Gelatin.**—The growth on this medium is much slower, more scanty, and less characteristic than that on the other media mentioned. This largely is on account of the lower temperature at which it must be used.

**Growth in Milk.**—The diphtheria bacillus grows readily in milk, beginning to develop at a comparatively low temperature (20° C.). The milk remains unchanged in appearance, as lactose is not fermented by the diphtheria bacillus.

**Pathogenesis.**—**In Lower Animals.**—The diphtheria bacillus, through its toxins is, when injected into their bodies, pathogenic for guinea-pigs, rabbits, chickens, pigeons, small birds, and cats; also in a lesser degree for dogs, goats, cattle, and horses, but hardly at all for rats and mice. In spite of its pathogenic qualities for these animals true diphtheria occurs in them with extreme rarity. As a rule supposed diphtheritic inflammations in them are due to other bacteria which cannot produce disease in man. The cat is the only animal that we have known to contract true diphtheria from contact with the disease. Cobbett reports a case in a colt. At the autopsy of animals dying from the poisons produced by the bacilli, the characteristic lesions described by Löffler are found. At the seat of inoculation there is a grayish focus surrounded by an area of congestion; the subcutaneous tissues for some distance around are edematous; the adjacent lymph nodes are swollen; and the serous cavities, especially the pleura and the pericardium, frequently contain an excess of fluid usually clear, but at times turbid; the lungs are usually congested, the suprarenals are markedly congested. In the organs are found numerous smaller and larger masses of necrotic cells, which are permeated by leukocytes. The heart and certain voluntary muscular fibers and nervous tissues usually show degenerative changes. Occasionally there is fatty degeneration of the liver and kidneys. The number of leukocytes in the blood is increased. From the area surrounding the point of inoculation virulent bacilli may be obtained, but in the internal organs they are only occasionally found, unless enormous numbers of bacilli have been injected. Paralysis, commencing usually in the posterior extremities and then gradually extending to the whole body and causing death by paralysis of the heart or respiration, is also produced in many cases in which the inoculated animals do not succumb to a too rapid intoxication. In a number of animals we have seen recovery take place three to six weeks after the onset of the paralysis.

**Tissue Changes in Natural (Human) Infection.**—The characteristic lesions are a pseudomembranous inflammation on some of the mucous membranes, or occasionally on the surface of wounds, and the general hyperplasias and parenchymatous inflammations produced by the absorbed toxic substances. Pneumonia is apt to occur as a complication of laryngeal diphtheria. The membrane may be simply a thin pellicle, which is easily removed without causing bleeding or it may be thick and firmly attached and leaving when removed a ragged bleeding surface. The tissue beneath the pseudomembrane is always intensely congested and often hemorrhagic. The cells show marked degenerative changes.

**Causes of Death.**—These are chiefly toxemia, laryngeal obstruction and bronchopneumonia. Septicemia due to other bacteria is frequently an additional factor.

**Diphtheria Toxin.**—This poison was assumed by Löffler (1884) to be produced by the bacilli, but it was first partially isolated by Roux and Yersin, who obtained it from cultures of the living bacilli by filtration through porous porcelain.

**The Production of Toxin in Culture Media.**—The artificial production of toxin from cultures of the diphtheria bacillus has been found to depend upon definite conditions, which are of practical importance in obtaining toxin for the inoculation of horses, and also of theoretic interest in explaining why cases of apparently equal local severity have such different degrees of toxic absorption. The researches of Roux and Yersin laid the foundation of our knowledge. Their investigations have been continued by Theobald Smith, Spronck, ourselves, and others. After an extensive series of investigations we (Park and Williams) came to the following conclusions: Toxin is produced by fully virulent diphtheria bacilli at all times during their life when the conditions are favorable. Under less favorable conditions some bacilli are able to produce toxin while others are not. Diphtheria bacilli may find conditions suitable for luxuriant growth, but unsuitable for the production of toxin. The requisite conditions for good development of toxin, as judged by the behavior of a number of cultures, are a temperature from about 32° to 37° C., a suitable culture medium, such as a 2 per cent. peptone nutrient bouillon made from veal, of an alkalinity which should be about 9 c.c. of normal soda solution per liter above the neutral point of litmus, and prepared from a suitable peptone (Witte) and meat. The culture fluid should be in comparatively thin layers and in large-necked Erlenmeyer flasks, so as to allow of a free access of air. The greatest accumulation of toxin in bouillon is after a duration of growth of the culture of from five to nine days, according to the peculiarities of the culture employed. At a too early period toxin has not sufficiently accumulated; at a too late period it has begun to degenerate. In our experience the amount of muscle sugar present in the meat makes no appreciable difference in the toxin produced when a vigorously growing bacillus is used, so long as the bouillon has been made sufficiently alkaline to prevent the acid produced by the fermentation of the sugar from producing in the bouillon an acidity sufficient to inhibit the growth of the bacilli. With the meat as we obtain it in New York, we usually get better results with unfermented meat than with fermented. In Boston, with the same bacillus, Smith gets his best results from the bouillon in which the sugar has been fermented by the colon bacillus. Instead of colon bacilli, yeasts may be added to the soaking meat, which is allowed to stand at about 25° C. The preliminary fermentation of the meat sometimes produces poisonous substances which are deleterious to the horses. We have obtained especially good results with veal broth made from calves two to four weeks old (bob veal). When strong toxin is desirable the muscle is separted from all fat, tendon and fibrous tissue before being chopped. (See page 130.)

Under the best conditions we can devise toxin begins to be produced by bacilli from some cultures when freshly sown in bouillon some time during the first twenty-four hours; from other cultures, for reasons not well understood, not for from two to four days. In neutral bouillon the culture fluid frequently becomes slightly acid and toxin production may be delayed for from one to three weeks. The greatest accumulation of toxin is on the fourth day, on the average, *after* the rapid production of toxin has commenced. After that time the number of living bacilli rapidly diminishes in the culture, and the conditions for those remaining alive are not suitable for the rapid production of toxin. As the toxin is not stable at 35° C., the deterioration taking place in the toxin already produced is greater than the amount of new toxin still forming.

Bacilli, when repeatedly transplanted from bouillon to bouillon, gradually come to grow on the surface only. This characteristic keeps the bacilli in contact with the oxygen and seems to aid in the development of toxin.

**Comparative Virulence as Estimated by Toxin Production of Different Cultures.**—The virulence of diphtheria bacilli from different sources, as measured by their toxin production, varies considerably. Thus, as an extreme instance, 0.002 c.c. of a forty-hour bouillon culture of our most virulent strain will kill a guinea-pig, which would require 0.1 c.c. of culture of our least virulent strain to kill. This difference frequently depends on the unequal growth of the bacilli, one culture having fifty times as many bacilli as the other. When the different strains are grown on ascitic broth, upon which their growth is usually good, the majority of cultures are nearly equal in virulence, but some still show marked differences. Moreover, the diphtheria bacilli differ somewhat in the tenacity with which they retain their power to produce toxin when grown outside the body. The bacillus that we have used to produce toxin in the laboratory of the Board of Health has retained its power unaltered for twenty-five years in bouillon cultures. Other bacilli have apparently lessened their capacity for toxin production after being kept six months. Brown and Smith[1] report on a culture in which 5 c.c. was the minimum toxic dose of the filtrate. The passage of diphtheria bacilli through the bodies of susceptible animals does not increase their toxic production to any considerable extent.

**Comparative Toxicity of Bacilli and Severity of Case.**—From the severity of an isolated case the toxicity of the bacilli cannot be determined. The presence of slight amounts of antitoxin in the blood of the person attacked and the association of other bacteria are at least two of the disturbing factors. The most toxic bacillus we have ever found was obtained from a mild case of diphtheria simulating tonsillitis. Another case, however, infected by this bacillus proved to be very severe. In localized epidemics the average severity of the cases probably indicates roughly the toxicity of the bacillus causing the infection, as here the individual susceptibility of the different persons infected would, in all likelihood, when taken together, be similar to that of other groups; but even in this instance special conditions of climate, food, or race may influence certain localities. It must be remembered that bacilli of like toxic power may differ in their liability to infect the mucous membrane; that is, the virulence of the organism may not go hand in hand with the toxicity.

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[1] Jour. Med. Res., xxx, No. 3, 443.

**Toxic Bacilli in Healthy Throats.**—Fully toxic bacilli have frequently been found in healthy throats of persons who have been brought in direct contact with diphtheria patients or diphtheria carriers without contracting the disease. It is therefore apparent that infection in diphtheria, as in other infectious diseases, requires not only the presence of toxic bacilli, but also a susceptibility to the disease, which may be local or general. We now know that 70 per cent. of all persons are protected from infection because of having antitoxin present in their blood. Among the predisposing influences which contribute to the production of diphtheritic infection may be mentioned the breathing of foul air and living in overcrowded and ill-ventilated rooms, impure food, certain diseases, more particularly catarrhal inflammations of the mucous membranes, and depressing conditions generally. Under these conditions an infected mucous membrane may become susceptible to disease. In connection with Beebe (1894) we made an examination of the throats of 330 healthy persons who had not come in contact, so far as known, with diphtheria, and we found toxic bacilli in 8, only 2 of whom later developed the disease. In 24 of the 330 healthy throats non-toxic bacilli similar to the toxic diphtheria bacillus were found. Very similar observations have since been made in Boston and by others in many widely separated countries. In 1905 von Sholly in our laboratory examined 1000 throats of those who had not knowingly been in contact with diphtheria and found toxic diphtheria bacilli in 0.5 per cent. of the cases. We have found toxic bacilli in about 5 per cent. of cases of scarlet fever. This indicates they are more prevalent in throats than a single culture from normal cases indicated.

**Persistence of Diphtheria Bacilli in the Throat.** — The continued presence of toxic diphtheria bacilli in the throats of patients who have recovered from the disease has been demonstrated by all investigators. In the investigations of 1894 we found that in 304 of 605 consecutive cases the bacilli disappeared within three days after the disappearance of the pseudomembrane; in 176 cases they persisted for seven days, in 64 cases for twelve days, in 36 cases for fifteen days, in 12 cases for three weeks, in 4 cases for four weeks, and in 2 cases for nine weeks. Since then we have met with a case in which they persisted with full toxicity for eight months. It is safe to say that in over 10 per cent. of the cases a few bacilli persist two weeks after the disappearance of the exudate and in over 1 per cent. four weeks. It is extremely difficult to prove that a case is absolutely clear, as the bacilli may remain hidden in the epithelial cells of some tonsillar crypt and not be detected by cultures.

**Classification of Carriers.**—Those harboring diphtheria bacilli after recovery from disease may be called convalescent carriers; those who carry the bacilli in their throats or adnexa though they have never given evidence of clinical diphtheria may be called contact carriers. These two groups may be transient carriers or chronic carriers. In chronic carriers the removal of hypertrophic lymphatic tissue aids the disappearance of the bacilli.

**Diphtheria-like Bacilli Not Producing Diphteria Toxin.**—In the tests of the bacilli obtained from hundreds of cases of suspected diphtheria which have been carried out during the past twenty years in the laboratories of the Health Department of New York City, in over 95 per cent. of cases the bacilli derived from exudates or pseudomembranes and possessing the characteristics of the Löffler bacillus have been found to be toxic, that is, producers of diphtheria toxin. But there are, however, in inflamed throats as well as in healthy throats, either alone or associated with the toxic bacilli, occasionally bacilli which, though morphologically and in their behavior on culture media identical with the toxic diphtheria bacillus, are yet producers, at least in artificial culture media and the usual test animals, of no diphtheria toxin. Between bacilli which produce a great deal of toxin and those which produce none we find a few minor grades of toxicity. We believe, therefore, in accordance with Roux and Yersin these non-toxic, but otherwise diphtheria-like, bacilli should be considered as possible attentuated varieties of the diphtheria bacillus which have lost their power to produce diphtheria toxin. This supposition is, however, not proved and it may be that the ancestors of these bacilli were never toxin producers. It is our belief that the great majority of these bacilli have lost permanently, if they ever had it, their pathogenic properties.

A number of observers have held, however, that all the various forms of diphtheria-like bacilli, even including the solidly-staining B. hofmanni (see below), are the result of more or less transitory variations of the same species and hence that the toxic forms are the result of a rapid adaptation to environment and consequent pathogenesis of the non-toxic forms, both typical and atypical. This question is of great practical importance in methods of handling the persons harboring these bacilli.

Wesbrook, Wilson and McDaniel[1] make a provisional classification based upon the morphology of the individual bacilli into three groups, called granular, barred and solid—two of the groups into seven types and the other into five, two of the types corresponding with those in the other groups not having been seen. They state that there is generally a sequence of types in the variations which appear throughout the course of the disease, the granular types, as a rule, predominating at the outset of the disease, and these giving place wholly or in part to the barred and solid types shortly before the disappearance of diphtheria-like organisms.

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[1] Tr. Assn. Am. Phys., 1900.

The inference drawn from this work is that the diphtheria bacillus may be rather easily, especially in the throat, converted into non-granular, solidly staining forms of the "pseudodiphtheria" type, and that the converse may occur, and that therefore all diphtheria-like bacilli must be considered a possible source of danger.

In studying the subject Williams[1] (1902) came to the following conclusions: Though some cultures change on some of the media, each changes in its own way, and each culture still has its distinct individuality. After many culture generations, especially when transplanted at short intervals, the different varieties of toxic diphtheria bacilli tend to run in lines parallel with a common norm, which seems to be a medium-sized, non-segmented bacillus, producing granules in early cultures on serum and growing well on all of the ordinary culture media. The non-toxic morphologically typical bacilli must be classed with the toxic varieties as one species, though there is little doubt that more minute study would show that the former constitute a distinct group. The atypical pseudo forms, however, which show no tendency to approach the norm of the typical forms, must be classed as distinct species. In studying successive direct smears from the throats of diphtheria patients no evidence of change from one type to the other was noted. Attempts have been made to give more virulence and some toxicity to a few of these varieties by successive peritoneal inoculations, and by growing the organisms in symbiosis with several other organisms, but in no instance has any increase of pathogenicity or decided change in morphological or cultural characteristics been noted.

Since there are so many different forms or varieties of diphtheria-like bacilli, it is quite possible that some of them are derived from strains of the diphtheria bacillus and that under certain conditions they regain the ability to produce toxin. This seems to be the only way to explain the results obtained by several observers. Such closely related varieties, however, do not appear to be common and we have up to the present been unable to obtain them. So we may safely say that in this region, at least, non-toxic diphtheria-like organisms retain their characteristics under various artificial and natural conditions, and that they may be regarded from a public health standpoint as harmless.

Among the non-toxic diphtheria-like bacilli which are obtained frequently from normal or slightly inflamed throats or from other mucous membranes are some that may be slightly pathogenic for guinea-pigs since they may kill, as we found, in doses of 5 c.c. of broth culture subcutaneously or intraperitoneally injected. Animals are not protected by diphtheria antitoxin from the action of these bacilli, showing that their poisonous action is not due to diphtheria toxin. At autopsy the bacilli are usually found more or less abundantly in the blood and internal organs. The fact that large injections of antitoxin serum hasten the death of guinea-pigs injected with these bacilli has given rise to the notion that injections of antitoxin might be dangerous in persons in whose throat these bacilli were present, either as saprophytes, or possibly, as inciters of slight disease. It is not the antitoxin, but the serum, which in large doses injures the vitality of the guinea-pigs and so slightly hastens death. The amount of serum required to produce this effect is far in excess of that properly given in man. These bacilli were first described by Davis[2] from our laboratory and later by Hamilton in 1904. The possibility of their being present affords no reason to avoid giving antitoxin in suspected cases. When pathogenic in man they are usually only feebly so.

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[1] Jour. Med. Res., June, 1902, viii, 83.

[2] Med. News, April 29, 1899.

**Resistance to Heat, Drying, and Chemicals.**—The thermal death-point of B. diphtheriæ with ten minutes' exposure is about 60° C., with five minutes 70° C. Boiling kills almost instantly. The bacillus has about the average resistance of non-sporebearing bacteria to disinfectants. In the dry state and exposed to diffuse light diphtheria bacilli usually die in from a few hours to a few days, but they may live for months; when in the dark, or protected by a film of mucus or albumin, they may live for even longer periods. Thus we found scrapings from dry bit of membrane to contain vigorous and toxic living bacilli for a period of four months after removal from the throat, and if the membrane had not been at that time completely used, living bacilli could probably have been obtained for a much longer period. On slate- and lead-pencils, toys, tumblers, as well as on paper money, they may live for several weeks, while on metal coins they die in twelve to thirty-six hours. In culture media, when kept at the blood heat, they usually die after a few weeks; but under certain conditions, as when sealed in tubes and protected from heat and light, they retain their life and toxicity for years. The bacillus is not sensitive to cold, for we found about 10 per cent. of the bacilli to retain their vitality and toxicity after exposure for two hours to several hundred degrees below zero. At temperatures just below freezing they may remain alive and toxic for a number of weeks.

**Transmission of Diphtheria.**—The possibility of the transmission of diphtheria from animals to man cannot be disputed; we have met two instances in which cats had malignant diphtheria, and many other animals can be infected, but, as we have said, there are few authentic cases of such transmission on record. So-called diphtheritic disease in animals and birds is usually, if not always, due to other microörganisms than the diphtheria bacilli.

The toxic bacilli have been found on soiled bedding or clothing of a diphtheria patient, or drinking-cups, candy, shoes, hair, slate-pencils, etc. These sources of infection by which the disease may be indirectly transmitted are, however, the less important ones. The usual source of the bacilli are the discharges of diphtheria patients; the secretions from the nose and throat of convalescent cases of diphtheria in which the toxic bacilli persist, and from the healthy throats of individuals who acquired the bacilli from being in contact with others having virulent germs. When we consider the number of healthy carriers and that it is only the severe types of diphtheria that remain isolated during their actual illness, the wonder is not that so many, but that so few, persons contract the disease.

**Susceptibility to and Immunity against Diphtheria.**—An individual susceptibility, both general and local, to diphtheria, as in all infectious diseases, is necessary to contract the disease. Age has long been recognized to be an important factor in diphtheria. Children within the first six months of life are but little susceptible, exceptionally, infants of a few weeks are attacked. The time of greatest susceptibility is between the second and tenth year. After the tenth year susceptibility decreases. Young animals born of mothers immune to diphtheria possess nearly the same degree of immunity as their mothers. They gradually lose this but retain traces up to six to twelve months. The human infant is now known to receive immunity from its mother. This immunity has been shown to depend in many on the presence of antitoxin.

**The Persistence in Man's Blood of Homologous and Alien Antitoxin.**—Antitoxins and other antibodies produced in an animal disappear more rapidly when introduced into the blood of another species than into one of the same species. In man an alien serum must be used in all except exceptional cases.

In our experiments in guinea-pigs we have found that the homologous antitoxin was retained in appreciable amounts for at least six months, while the heterologous antibodies were noticeable to the same extent for only three weeks. There is a rather rapid loss of horse-produced antitoxin during the first few days and then a slow loss, becoming more and more gradual until final elimination at the end of ten days to three weeks. The larger the amount of antibodies injected the longer will be the time before the elimination of effective amount.

For a discussion of the nature of antitoxin see page 180. For a presentation of the therapeutic use of antitoxin see Part III.

**Relation of Bacteriology to Diagnosis.**—We believe that all experienced clinicians will agree that there are certain mild exudative inflammations of the throat of similar appearance some of which transmit diphtheria while others do not.

The doubtful cases that have the diphtheria bacilli in the exudate, are capable of giving true characteristic diphtheria to others, or later develop it characteristically themselves, while those in whose throats no diphtheria bacilli exist can under no condition give true characteristic diphtheria to others or develop it themselves. It is, indeed, true, as a rule, that cases presenting the appearance of ordinary follicular tonsillitis in adults are not due to the diphtheria bacillus and do not harbor these bacilli. On the other hand, in small children mild diphtheria very frequently occurs with the semblance of rather severe ordinary follicular tonsillitis, due to the pyogenic cocci; and in large cities where diphtheria is prevalent all such cases must be watched as being more or less suspicious. Most observers agree with us in thinking that if in any case exposure to diphtheria is known to have occurred, even a slightly suspicious sore throat should be regarded as probably due to the diphtheria bacilli. If, on the other hand, no cases of diphtheria have been known to exist in the neighborhood, even cases of a more suspicious nature would probably not be regarded as diphtheria. Now that we know about 50 per cent. of children have a fairly constant supply of antitoxin in their blood, we have reason to believe that many of these doubtful cases are simply diphtheria carriers in which the bacilli are taking little or no part in making the lesions. Like any carriers they are dangerous to others.

The presence of irregular-shaped patches of adherent grayish or yellowish-gray pseudomembrane on some other portions than the tonsils is, as a rule, an indication of the activity of the diphtheria bacilli. Restricted to the tonsils alone, their presence is less certain.

Occasionally, in scarlatinal angina or in severe phlegmonous sore throats, patches of exudate may appear on the uvula or borders of the faucial pillars, and still the case may not be due to the diphtheria bacilli; these are, however, exceptional. Thick, grayish pseudomembranes which cover large portions of the tonsils, soft palate, and nostrils are almost invariably the lesions produced by diphtheria bacilli.

The very great majority of cases of pseudomembranes of exudative laryngitis, in the coast cities at least, whether an exudate is present in the pharynx or not, are due to the diphtheria bacilli. Nearly all membranous affections of the nose are true diphtheria. When the membrane is limited to the nose the symptoms are, as a rule, very slight; but when the nasopharynx is involved the symptoms are usually grave.

Many cases of pseudomembranes and exudates, entirely confined to portions of the tonsils in adults, are not due to the diphtheria bacilli. Cases presenting the appearances found in scarlet fever, in which a thin grayish membrane lines the borders of the uvula and faucial pillars, are rarely diphtheritic. As a rule pseudomembranous inflammations complicating scarlet fever, syphilis and other infectious diseases are due to the activity of the pathogenic cocci and other bacteria, induced by the inflamed conditions of the mucous membranes due to the scarlatinal or other poison. The possibility of these persons being carriers of diphtheria bacilli must always be kept in mind.

Paralysis following a pseudomembranous inflammation is an almost positive indication that the case was one of diphtheria, although slight paralysis has followed in a very few cases in which careful cultures have revealed no diphtheria bacilli. These, if not true diphtheria, must be considered very exceptional cases.

**Bacteriological Diagnosis.**—From the above it is apparent that fully developed characteristic cases of diphtheria are readily diagnosticated, but that many of the less marked, or at an early period undeveloped, cases are difficult to differentiate. In these cases cultures are of the utmost value, since they enable us to isolate those in which diphtheria-like bacilli are found, and to give preventive injections of antitoxin to both the sick and those in contact with them, if this has not already been done. As a rule cultures do not give us as much information as to the gravity of the case as the clinical appearances, for before the lapse of the twelve hours required for the laboratory report, the extent of the disease usually allows a prognosis. The reported absence of bacilli in a culture must be given weight in proportion to the skill with which the culture was made, the suitableness of the media, the location of the disease, and the knowledge and experience of the one who examined it.

Diphtheria does not occur without the presence of the diphtheria bacilli; but there have been many cases of diphtheria in which, for one or another reason, no bacilli were found in the cultures by the examiner. In many of these cases later cultures revealed them. The reverse is also true, the presence of diphtheria bacilli in throats without clear clinical signs of diphtheria in no sense makes it a case of diphtheria. In a convalescent case the absence of bacilli in any one culture indicates that there are certainly not many bacilli left in the throat, but even repeated cultures cannot absolutely prove their total absence, for in some deep tonsillar crypt a few bacilli may remain in the epithelial cells. The physician must have intelligence to use advantageously laboratory findings.

**Technic of the Bacteriological Diagnosis.**—*Collection of the Animal Blood Serum and its Preparation for Use in Cultures.* (See page 122.)

*Swab for Inoculating Culture Tubes.*—The swab we prefer to use to inoculate the serum is made as follows: A stiff, thin, iron rod, six inches in length, is roughened at one end by a few blows of a hammer, and about this end a little absorbent cotton is firmly wound. Each swab is then placed in a separate glass tube, and the mouths of the tubes are plugged with cotton. The tubes and rods are then sterilized by dry heat at about 150° C. for one hour, and stored for future use. These iron rods have proved more serviceable for making inoculations than platinum-wire needles or wooden sticks, especially in young children and in laryngeal cases. It is easier to use the cotton swab in such cases and it gathers up so much more material for the inoculation that it has seemed more reliable. The wood unless very carefully selected is apt to break and is too thick to use in the nose.

For convenience and safety in transportation "culture outfits" have been devised, which consist usually of a small wooden box containing a tube of blood serum, a tube holding a swab and a record blank. These "culture outfits" may be carried or sent by messenger or express to any place desired.

*Directions for Inoculating Culture Tubes with the Exudate.*—The patient is placed in a good light, and, if a child, properly held. The swab is removed from its tube, and, while the tongue is depressed with a spoon, is passed into the pharynx (if possible, without touching the tongue or other parts of the mouth), and is rubbed gently but firmly against any visible membrane on the tonsils or in the pharynx, and then, without being laid down, the swab is immediately inserted in the blood-serum tube, and the portion which has previously been in contact with the exudate is rubbed a number of times back and forth over the whole surface of the serum. This should be done thoroughly, but it is to be gently done, so as not to break the surface of the serum. The swab should then be placed in its tube, and both tubes, thin cotton plugs having been inserted, are reserved for examination or sent to the laboratory or collecting station (as in New York City). If sent to the Health Department laboratories for examination the blank forms of report which usually accompany each "outfit" should be filled out and forwarded with the tubes.

Where there is no visible membrane (it may be present in the nose or larynx) one swab should be rubbed over the mucous membrane of the pharynx and tonsils, and another in the nasal cavities, and a culture made from these. In very young children it should be remembered that the throat often contains food or vomited matter. This should be cleared away before using the swab to make the bacteriological examination easier. Under no conditions should any attempt be made to collect the material shortly after the application of strong disinfectants (especially solutions of corrosive sublimate) to the throat. Cultures from the nostrils are often more successful if the nostrils are first cleansed with a spray of sterile normal salt solution.

*Examination of Cultures.*—The culture tubes which have been inoculated, as described above, are kept in an incubator at 37° C. for at least twelve hours and are then ready for examination. When great haste is required, even five hours will often suffice for a sufficient growth of bacteria for a skilled examiner to decide as to the presence or absence of the bacilli. The absence of bacilli at this period cannot be relied upon. In primary cultures it is wise to reincubate tubes taken out under sixteen hours in which no bacilli were found. A small percentage of these will yield positive results after a few hours' further incubation. On inspection it will be seen that the surface of the blood serum is dotted with numerous colonies, which are just visible. No diagnosis can be made from simple inspection; if, however, the serum is found to be liquefied or shows other evidences of contamination the examination will probably be unsatisfactory.

In order to make a microscopic preparation, a clean platinum needle is inserted into the tube and quite a large number of colonies are swept with it from the surface of the culture-medium, a part being selected where the most suitable colonies are found. A sufficient amount of the bacteria adherent to the needle is washed off in a tiny droplet of water previously placed on the glass slide and smeared over its surface. The bacteria on the glass are then allowed to dry in the air. The glass slide is then passed quickly through the flame of a Bunsen burner or alcohol lamp, three times in the usual way, covered with a few drops of Löffler's solution of alkaline methylene blue, and left without heating for five to ten minutes. It is then rinsed off in clear water, dried, and mounted in balsam. When other methods of staining are desired they are carried out in the proper way (see Methods of Staining).

In the great majority of cases one of two pictures will be seen with the $\frac{1}{12}$ oil-immersion lens—either an enormous number of characteristic Löffler bacilli, with a moderate number of cocci, or a pure culture of cocci, mostly in pairs or short chains. (See Streptococcus.) In a few cases there will be an approximately even mixture of Löffler bacilli and of cocci, and in others a great excess of cocci. Besides these there will be occasionally met preparations in which, with the cocci, there are mingled bacilli more or less resembling the Löffler bacilli. These bacilli, which are usually of the pseudodiphtheria type of bacilli (see Fig. 128), are especially frequent in cultures from the nose.

In not more than one case in twenty will there be any serious difficulty in making the diagnosis, if the serum in the tube is moist and has been properly inoculated. In the doubtful case another culture must be made or the bacilli plated out and tested in pure culture.

*Direct Microscopic Examination of the Exudate.*—An immediate diagnosis without the use of cultures is often possible from a microscopic examination of the exudate. This is made by smearing a slide or cover-glass with a little of the exudate from the swab, drying, heating, staining, and examining it microscopically. This examination, however, is much more difficult, and the results are more uncertain than when the slides are prepared from cultures. The bacilli from the membrane are usually less typical in appearance than those found in cultures, and they are mixed with fibrin, pus, and epithelial cells. They may also be very few in number in the parts reached by the swab, or bacilli may be found which closely resemble the Löffler bacilli in appearance, but which differ greatly in growth and in other characteristics, and have absolutely no connection with them. When in a smear containing mostly cocci a few of these doubtful bacilli are present, it is impossible either to exclude or to make the diagnosis of diphtheria with certainty. Although in some cases this immediate examination may be of the greatest value, it is not a method suitable for general use, and should always be controlled by cultures. When carried out in the best manner an experienced bacteriologist may obtain markedly accurate results. Higley, in a series of consecutive throat cases, made the same diagnosis from the direct examination of smears as the Health Department laboratory made from the culture. To get the exudate he used a probe armed with a loop of heavy copper wire which had been so flattened as to act as a blunt curette. He then made thin smears from the exudate. After drying and fixing by heat the smears were stained for five seconds in a solution made by adding five drops of Kühne's carbolic methylene blue to 7 c.c. of tap-water. After washing and drying they were stained for one minute in a solution of 10 drops of carbol-fuchsin in 7 c.c. of water. The dilute solution should be freshly prepared. The diphtheria bacilli will appear as dark red or violet rods, and their contour, mode of division, and arrangement are manifest.

**Animal Inoculation as a Test of Toxicity.**—*The So-called Virulence Test.*—It must be remembered that the above described method if positive, only demonstrates the presence of bacilli having the morphology of diphtheria bacilli. Where a typical membranous inflammation exists, actual tests, as we have said, have shown that the bacilli are almost always true diphtheria bacilli, therefore for practical purposes they should be considered such and for quarantine purposes the cultures repeated until, as will occur in the majority, bacilli disappear. If positive cultures continue, or if it is a question of a normal carrier, a complete identification should be carried out, to release from quarantine those who may be harboring only harmless diphtheria-like bacilli. This determination is spoken of as the "virulence test," or the test for specific toxin production, as the final identification of a suspected diphtheria bacillus rests on the demonstration that it produces a toxin which is neutralized by known diphtheria antitoxin.

*Technic of Virulence Test.*—The first step in the test is the isolation of a pure culture of the bacillus. A number of plates are poured using nutrient agar or ascitic infusion agar (one part of ascitic fluid to five of agar). After hardening, the surface is streaked across with the growth from the Löffler tube, selecting when possible colonies which, in color and size, resemble those of the diphtheria bacillus and also using a general mixture preferably from the drier portions of the serum medium. After incubation for sixteen hours at 37° C., the growth developing along the line of streak should be examined with the microscope using a low-power objective preferably a No. 2. Diphtheria colonies are most likely to be found at the edges of the streak. These are fished in the usual way by inserting a straight wire between the objective and the agar, the path of the wire and the actual touching of the correct colony being observed with the microscope. Care must be taken that the wire does not touch anything but the colony. The growth is transferred to ascitic fusion broth or Löffler medium depending on the subsequent method of test selected.

If very few bacilli are present in the original culture inoculations should be made into several tubes of ascitic broth. As the diphtheria bacillus will grow mostly on the surface of such broth, this portion can be used to inoculate plates with success where direct plates fail.

After incubation, the pure cultures are examined by smear to determine whether diphtheria-like bacilli are present. If the examiner has some experience, he can exclude some cultures at once because of their atypical morphology, especially the short solidly staining B. hofmanni. If in doubt, inoculation of glucose-media can be resorted to as a further procedure, and in differentiation, since many of these atypical types fail to produce acid in glucose media, whereas B. diphtheriæ does.

After determining that the cultures are pure and morphologically typical, animal inoculation is resorted to. Two methods are available, the subcutaneous and the intracutaneous, *The Subcutaneous Method.*—In this method, 1 c.c. of the ascitic broth culture (incubated forty-eight hours) is injected subcutaneously into a guinea-pig, about 250 gms. in weight. A second guinea-pig is injected with same amount of culture to which had been added fifty to a hundred units of diphtheria antitoxin. If the first pig dies within two to three days and on postmortem, shows the typical engorgement of the suprarenals, and local edema already described and the second pig lives, the bacillus is a true toxin producing diphtheria bacillus, otherwise it is not.

*The Intracutaneous Method.*—The intracutaneous method requires some experience in recognizing the skin lesions. Its advantage is that four to six cultures can be tested with only two pigs.[1] The abdomens of the pigs are denuded of hair by pulling it out. This is done by grasping an area of hair between thumb and finger and exerting a jerk-like pull. Diphtheria antitoxin, about 200 units, is administered to the control pig by intraperitoneal injection. The growth from a Löffler slant is rubbed up in 20 c.c. of salt solution and 0.15 c.c. injected intracutaneously into both pigs. The culture used must not be over twenty-four hours old. If the bacillus is a true diphtheria bacillus, it will develop toxin which will produce in the non-immunized pig a definite local inflammatory lesion after twenty-four hours which goes on to superficial necrosis in forty-eight to seventy-two hours. The pig receiving antitoxin will show no lesion.

Although a very few true diphtheria bacilli have been encountered which produce too little toxin to be demonstrable by these methods, they are so infrequent that for practical purposes they can be ignored. If the virulence test is negative, the case may be discharged from quarantine.

**The Schick Reaction.**—Schick[2] has published a method by which the presence of antitoxin in the blood and tissue can be determined very easily. A minute quantity of toxin is injected intracutaneously, and a local reaction follows if there is less than $\frac{1}{30}$ of a unit of antitoxin per cubic centimeter of blood. This amount is considered sufficient to protect against diphtheria. The explanation of the test is that when no antitoxin is present the toxin acts on the skin; when antitoxin is present it neutralizes the toxin so that no poisoning occurs. A negative reaction therefore indicates the presence of antitoxin.

A standard diphtheria toxin is diluted at first 1 to 10 in 0.5 per cent. phenol; this dilution will keep in the ice-box with little deterioration for at least two weeks. For use further dilutions are made in normal saline, of such strength, that 0.1 c.c. contains $\frac{1}{50}$ of the minimum lethal dose for the guinea-pig. This amount is injected intracutaneously on the flexor surface of the arm or forearm. If the injection has been made properly a definite wheal appears which lasts for several minutes. It is of the utmost importance that the injection be made in and not under the skin. Some persons prefer such a dilution that 0.2 c.c. contains the dose. This dilution gives a less intense reaction than the stronger solution.

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[1] Rabbits may also be used.

[2] München. med. Wchnschr., 1913, ix, 2608–2610.

A positive reaction appears in twenty-four to thirty-six hours in the majority of cases, but in some it is delayed as late as seventy-two hours, and is characterized by a circumscribed area of redness and slight infiltration which measures from 1 to 2 cm. in diameter. It persists for seven to fifteen days, and on fading shows superficial scaling and a persistent brownish pigmentation. The test represents a true irritant action of non-neutralized toxin. Pseudoreactions are seen occasionally in young children and rather frequently in adults. They are absent in very young infants. These are local sensitization phenomena of a general protein character, and can usually be distinguished from the true reaction due to the specific toxin. They appear earlier, are more infiltrated, less sharply circumscribed and usually disappear in twenty-four to seventy-two hours. On fading they leave a faintly pigmented area which may show superficial scaling. They can also be obtained with the neutralized toxin and in some with dilutions of plain broth.

By the use of this test a large number of individuals have been shown to be naturally immune. By applying the test, therefore, passive immunization with antitoxin of a large number of individuals can be omitted and the disagreeable symptoms of sensitization avoided. Of 400 cases of scarlet fever isolated in an hospital for contagious diseases, showing a negative Schick reaction and receiving no immunizing dose of antitoxin, not one developed clinical diphtheria. Of these cases many were carriers of virulent diphtheria bacilli.

Our results closely parallel those of Schick. In various groups we have obtained quite different results as shown in the table.

SUMMARY OF SCHICK TESTS SHOWING MINIMUM AND MAXIMUM PERCENTAGE OF SCHICK REACTIONS.

| | Per cent. +Shick. |
|---|---|
| 1 to 2 years | 50 to 70 |
| 2 to 4 years | 32 to 60 |
| 4 to 6 years | 25 to 55 |
| 6 to 8 years | 21 to 55 |
| 8 to 10 years | 22 to 55 |
| 10 to 12 years | 21 to 50 |
| 12 to 14 years | 17 to 50 |
| 14 to 16 years | 16 to 50 |
| 16 to 30 years | 15 to 40 |
| | 21 to 50 |

From these figures, then, we may assume that the percentage of individuals susceptible to diphtheria is greatest between the ages of one to four years. When a person once develops antitoxin it is apt to be a permanent immunity. Very few giving a negative Schick ever change. Schick reported positive reactions in 7 per cent. of the newborn, in 43 per cent. during the second six months of life, in 60 per cent. between one and five years and 50 per cent. between five and fifteen years.

**Toxin-antitoxin Inoculations.**—Behring was the first who brought about the practical application in children for purposes of immunization against diphtheria of toxin-antitoxin mixtures in which the toxin had been so neutralized as to be no longer poisonous. The use of this immunizing agent is the result of a long series of investigations as to the possibility of producing antitoxin through injection of these mixtures. In 1895 Babes carried out successful experiments in guinea-pigs. Since 1897 the horses in the Health Department of New York City, which are used to produce diphtheria antitoxin, have been immunized at first with these neutral mixtures. In 1903 one of us published the records of a number of horses showing that three injections might cause production of several hundred units of antitoxin in each cubic centimeter of serum. Theobald Smith later made a careful study of the subject in guinea-pigs and suggested the toxin-antitoxin injections in children for practical immunization but never tried it.

Behring deserves, therefore, the credit not of the discovery of the method but of actually applying the toxin-antitoxin mixtures for the immunizing of persons against diphtheria. His investigations were left incomplete because of the war.

We have personally watched the result in a series of over 4000 cases, that had been actively immunized with diphtheria toxin-antitoxin. These susceptible individuals were selected by means of the Schick test out of a total of about 10,000 infants, children and adults in 10 different institutions.

The mixtures of toxin-antitoxin that were used for immunization contained about 2L+ doses of toxin to each cubic centimeter, and were either neutral (66–70 per cent. L+ to each unit of antitoxin) or slightly toxic (80–90 per cent. L+ to each unit of antitoxin) to the guinea-pig. The dose was varied from 0.5 c.c. to 1.0 c.c., and the number of injections from one to three. The injections were made subcutaneously at intervals of seven days. The local reactions at the site of injection were generally mild; in the older children and adults, the redness and swelling were more marked. General symptoms, like malaise, and temperature of 100° to 102° F., were noted in 10 to 20 per cent. of the cases; in a few the temperature reached 104° F. The symptoms lasted twenty-four to forty-eight hours, and then rapidly subsided. Both local and general symptoms were especially evident in some of those who showed a susceptibility to the protein by giving a combined pseudo and true Schick reaction. No harmful after-effects were noted except in twelve cases in which superficial abscesses developed. All made a good recovery.

The retests with the Schick reaction showed that only 30 to 40 per cent. became immune three weeks after the first injection; about 50 per cent. at four weeks, 70 to 80 per cent. at six weeks, and 85 to 90 per cent. at eight to twelve weeks. The best results were obtained with the full immunization, consisting of three injections of 1 c.c. each, given at weekly intervals. The duration of the active immunity was studied in a group of children that was followed up for over three and one-half years; these cases showed that the active immunity persisted for at least that length of time. It is possible that the immunity induced by the injections of toxin-antitoxin starts a continued cellular production of antitoxin which otherwise would have appeared much later in life.

From their results Park and Zingher[1] conclude that it is advisable to immunize children soon after the first year of life so as to afford them a protection against diphtheria at a time when the disease is most dangerous. In addition such young children, by not having any hypersensitiveness to the bacillus protein, show very mild local and constitutional symptoms after the injections. An immune child population could thus be developed with the result that fresh clinical cases would be prevented and the bacillus-carrier menace would probably soon disappear as a hygienic factor in our communities. Under our supervision Miss Denny watched the effect of the injections in two thousand infants none of whom were over one week old. Absolutely no bad effects were noted. The immunity response is somewhat less than in older children, but it appears that about 80 per cent. remain immune after the time when the passive immunity derived from the mother usually disappears. Under the supervision of Dr. Schroder the school children in the primary grades of twenty public schools were Schick tested and those giving a positive reaction injected with two toxin-antitoxin mixtures. It is intended to extend this work in the fall of 1919.

Interesting and parallel results to those in human beings were noted in guinea-pigs and horses. *Guinea-pigs* have no normal antitoxin and are fairly resistant to active immunization with diphtheria toxin-antitoxin, and in that respect they show an almost complete parallellism to the positive Schick cases among human beings. After injections of toxin-antitoxin, an antitoxic immunity develops slowly from the sixth to eighth week. *Horses*, on the other hand, as a rule, correspond to those human beings who are naturally immune in their behavior toward small doses of toxin-antitoxin. They both give a ready response, even after a single injection of toxin-antitoxin, and show a distinct increase in the antitoxin content toward the end of the first week. Occasionally a horse is found that has no antitoxin in the control bleeding; such animals respond slowly to small doses of toxin-antitoxin. It is probable that the tissue cells of the naturally immune human beings and the majority of horses have acquired the property of giving a quick and easy response to the stimulation of diphtheria toxin.

**Mixed Infection in Diphtheria.**—Toxic diphtheria bacilli are not the only pathogenic bacteria present in human diphtheria. Various cocci and bacilli more particularly streptococci, staphylococci, pneumococci, and influenza bacilli, are always found actively associated with Löffler's bacillus in diphtheria, playing an important part in the disease and leading often to serious complications (sepsis and bronchopneumonia). Investigations indicate that when other pathogenic bacteria are associated with diphtheria bacilli they mutually assist one another in their attacks upon the mucous membrane, the streptococcus being particularly active in this respect, often opening the way for the invasion of the Löffler bacillus into the deeper tissues or supplying needed conditions for the development of its toxin. In most fatal cases of bronchopneumonia following laryngeal diphtheria we find not only abundant pneumococci or streptococci in the inflamed lung areas, but also in the blood and tissues of the organs. As these septic infections due to the pyogenic cocci are in no way influenced by the diphtheria antitoxin, they frequently are the cause of the fatal termination. Other bacteria cause putrefactive changes in the exudate, producing alterations in color, *e. g.*, B. pyocyaneus, and offensive odors, *e. g.*, B. fusiformis.

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[1] Archive. Pediatrics, 1914.

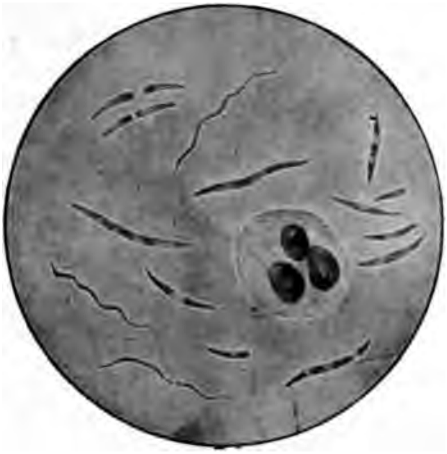

FIG. 127.—Vincent's bacillus (B. fusiformis) with accompanying spirochetes.

**Pseudomembranous Exudative Inflammations Due to Bacteria other than the Diphtheria Bacilli.**—The diphtheria bacillus, though the most usual, is not the only microörganism that is capable of producing pseudomembranous inflammations. There are numerous bacteria present almost constantly in the throat secretions, which, under certain conditions, can cause local lesions very similar to those in the less marked cases of true diphtheria. The streptococcus and pneumococcus are the two forms most frequently found in these cases, but there are also others, such as Vincent's bacillus (see Fig. 126 and p. 501), which, under suitable conditions, excite this form of inflammation, but without serious constitutional symptoms.

The pseudomembranous angina accompanying scarlet fever, and to a less extent other diseases, may not show the presence of diphtheria bacilli, but only the pyogenic cocci, especially streptococci, or, more rarely, some varieties of little-known bacilli. The deposit covering the inflamed tissues in these non-specific cases is, it is true, usually but not always, rather an exudate than a true pseudomembrane.

**Diphtheroids.**—There are a large number of small more or less irregular bacilli bearing some morphological resemblance to the diphtheria bacillus which have been loosely termed diphtheroids. They have been found in the nose, throat, eye and other parts of the body, both in health and in disease (Hodgkin's disease, leukemia, etc.). Several of them, *e. g.*, *B. hofmanni*, *B. xerosis*, are considered distinct species (see below).

Since this type of organism is so widely distributed, any specific pathogenic properties attributed to them, unless positive proof is offered, must be received with reserve.

**Bacillus Hofmanni (Pseudodiphtheria Bacilli).**—These bacilli are rather short, plump, and more uniform in size and shape than the true Löffler bacillus (Fig. 128). On blood serum their colony growth is very similar to that of the diphtheria bacilli. The great majority of them in any young culture show no polar granules when stained by the Neisser method, and stain evenly throughout with the alkaline methylene blue solution. They do not produce acid by the fermentation of glucose, as do all known virulent and many non-virulent diphtheria bacilli; therefore there is no increase in acidity in the bouillon in which they are grown during the first twenty-four hours from the fermentation of the meat sugar regularly present. They are found in varying abundance in different localities in New York City, in about 1 per cent. of the normal throat and nasal secretions, and seem to have now at least no connection with diphtheria; whether they were originally derived from diphtheria bacillus is doubtful. They have been called *pseudodiphtheria bacilli*, and more properly *B. hofmanni*. In bouillon they grow, as a rule, less luxuriantly than the diphtheria bacilli. Some of the varieties of the pseudodiphtheria bacilli are as long as the shorter forms of the virulent bacilli. When these are found in cultures from cases of suspected diphtheria they may lead to an incorrect diagnosis. These bacilli are found occasionally in all countries where search has been made for them. There are also some varieties of diphtheroids which resemble the short *B. hofmanni* in form and staining, but which produce acid in glucose bouillon.

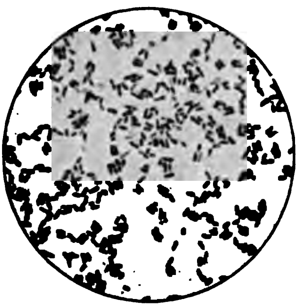

FIG. 128.—Pseudodiphtheria bacilli. (B. hofmanni.)

**Bacillus Xerosis.**—Diphtheria-like bacilli were found by Kutschert and Neisser (1884) in the condition known as xerosis conjunctivæ. Since then they have been found frequently in normal conjunctivæ. These bacilli went by the name of xerosis bacilli. Under this name, no doubt, different observers have placed several varieties of bacilli morphologically somewhat similar to the diphtheria bacilli. Knapp first limited the name to bacilli that ferment saccharose and not dextrin, in contradistinction to diphtheria bacilli which ferment dextrin and not saccharose. The fermentation tests for three of the diphtheria group, according to Knapp, may be shown as follows:

| Species. | Dextrose. | Levulose. | Galactose. | Maltose. | Saccharose. | Dextrin. |
|---|---|---|---|---|---|---|
| B. diphtheria | + | + | + | + | − | + |
| B. xerosis | + | + | + | + | + | − |
| B. hofmanni | − | − | − | − | − | − |

NOTE.—One per cent. sugar in Hiss serum-water media.

These reactions do not separate a large number of the non-virulent bacilli morphologically like the diphtheria bacilli mentioned before.

**B. Segmentosus.**—Cantley,[1] in England, in 1893, described a bacillus which he found in acute rhinitis and called B. caryzæ segmentosus. It has since been found in acute rhinitis in a large percentage of cases by Burnham, Allen[2] and others in England and Walter[3] in this country. It is characterized by a barring due to an unstained septum. It is known in England as B. septus. It has not been minutely enough studied to differentiate it from B. xerosis.

**Micro-aërophilic "Diphtheroids."**—Certain "diphtheroid" bacilli chose for their most abundant growth that part of the medium which contains only a small amount of oxygen. Thus in a deep shake agar tube culture, a band of growth appears a short distance from the surface of the medium and few colonies appear below this band. Among these microaërophilic bacilli (see page 54) the acne bacillus is of interest to us because of its probably etiological importance in acne vulgaris.

**Bacillus Acne.**—A short, rather plump, irregularly shaped, bacillus was first reported as occurring in acne pustules and comedones by Unna[4] in 1894. A morphologically similar bacillus was grown by Sabouraud, in 1897, in pure cultures on an acid glycerin agar. Whether or not this was the acne bacillus remains doubtful, since pure aërobic cultures are so difficult to obtain. In 1907 Halle and Civatte[5] found an anaërobe in the sebaceous follicles from all faces examined. This threw doubt on the etiological relationship of this bacillus to acne. But Gilchrist and others found a similar bacillus most abundantly in acne lesions, though it was frequently accompanied by a small coccus. Fleming,[6] in 1909, found a similar anaërobic diphtheroid in large numbers in acne vulgaris and obtained abundant anaërobic growths on oleic acid glycerin agar.

The bacillus now accepted as *B. acne* has the following characteristics: The cultures are made up of short, rather irregularly club-shaped, non-motile rods averaging $2\mu$ by $0.5\mu$. These are Gram-positive and take ordinary stains well. They grow best with only a small amount of oxygen; that is, in shake tube cultures the most abundant growth appears in the form of a hazy band about half an inch from the surface of the medium and about a fourth of an inch thick. This develops in about three to five days at 36° C.

In our laboratory a semisolid medium (page 116) has been found to be best for the isolation of this bacillus. A shake culture is made in a tube of this medium. If the tube, after sterilization by carbolic acid, is cut just below the zone of growth and new shake culture tubes made from a portion taken from the periphery of this zone, repeating on subcultures if necessary, a pure culture will finally result.

The question of the efficacy of vaccine treatment in acne vulgaris with *B. acne* and with *Staphylococcus pyogenes* is taken up in the chapter on the Therapeutic Application of Vaccines in Section III.

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[1] Local Government Reports, 1894, 5.

[2] Lancet, November, 1908, p. 1591.

[3] Jour. Am. Med. Assn., 1910, lv, 1091.

[4] Histopathology of Diseases of the Skin, 1894.

[5] Ann. de Dermat. et de. Syph., 1907, 184.

[6] Lancet, 1909, i, 1207.

# CHAPTER XXI.

# MICROÖRGANISMS OF THE ALIMENTARY CANAL AND THEIR CONTROL.

**Conditions Influencing Microbal Development.**—The alimenary tract both as regards foodstuff, varying degrees of oxygen tension and reaction gives so many different conditions for growth that many varieties of microörganisms find an optimum environment at some level. Thus, the mouth offers, in general, an aërobic area, although with its various crevices between the teeth, in the folds of the mucous membranes and the crypts of the tonsils, anaërobic types may flourish with aërobic types, which absorb the oxygen. The stomach when its acidity is normal, destroys most germs unless protected by food particles. As soon as the duodenum is reached the oxygen tension becomes low and from there on an anaërobic condition exists. More aërobic conditions are reached in the lower colon and rectum. It is evident that the degree of digestion of the food and the proportion of unabsorbed carbohydrate and protein at different levels will also influence the flora, likewise the products of bacterial growth at one level will influence the growth of other types at this and at lower levels. As the intestinal contents pass downward, they carry with them the flora from a higher level, but if the conditions are not favorable for their growth they are quickly overshadowed by the more adapted types. In the lower part of the large intestine and in the rectum due to the gradual loss of water, there is marked tendency of the microörganisms in fecal mass to die or to become so attenuated as to be incapable of further growth. It is estimated that at least 5 to 8 grams of bacteria, mostly dead, are excreted each day with the feces.

**Development of the Intestinal Flora.**—At birth the meconium is sterile unless fetal infection has taken place due to general infection in the mother. Shortly after birth bacteria and yeasts are found entering either through the rectum or by way of the mouth from swallowing saliva or food. Very soon, through further ingestion of microbes and by multiplication of different types at their optimum levels, a more or less distinctive alimentary flora is established, distinctive in that the breast-fed infant, the artificially-fed infant and the adolescent or adult each has, considering the dominant types, a somewhat characteristic flora. This flora is susceptible, however, to variation through the administration of cathartics, by changes in diet, by implantation through feeding of cultures or during alimentary infections. The possibility of implantation of microörganisms by feeding is considered below.

**Importance of the Intestinal Flora.**—Whether the development of such an intestinal flora is of physiological advantage to the host is open to question. Successful experiments in raising animals with a sterile intestinal tract show that such a flora is not a physiological necessity. Experiments in which animals so raised did not do well cannot be considered evidence against this view, as the conditions of the experiment may have deleteriously influenced development. Undoubtedly the character and balance of the flora does act as a protection under some circumstances, being antagonistic to the implantation of an exogenous pathogenic type. Abnormal variations, however, in balance between the dominant types may be the basis of intestinal symptoms (see below). In one sense, part of the intestinal flora is a potential menace to the host. The intestinal mucosa is a partial obstacle to the passage of the microbes into the tissues, lymph, and blood. A few, however, evidently escape as the fairly frequent localization in the gallbladder, kidney and elsewhere show. Probably this occurs constantly in small numbers, but because of the slight virulence of the majority of such types or due to the resistance of the host or both they are promptly disposed of.

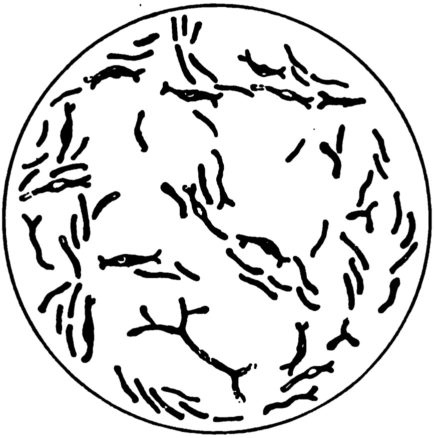

FIG. 129.—B. bifidus, representing the various forms described; the irregularly stained or vesicular forms being from old cultures. × about 1800 diameters.

**Dominant Intestinal Bacterial Types.**—**B. bifidus.**—A strict anaërobe isolated by Tissier from the stools of breast-fed infants and from the superficial ducts of the mammary glands of mothers. It is Gram-positive or contains a Gram-positive granule, the remainder of the bacillus being Gram-negative. In stools it is a slender bacillus with one end tapering, the other club-shaped. In cultures it has the property of developing bifid ends. It produces acid freely but no gas from lactose or other sugars.

**B. Acidophilus.**—A type of bacterium characterized by its acid tolerance. B. bulgaricus (see below) is related to this type, both being included in the aciduric group. It is a Gram-positive, non-sporebearing pleomorphic bacillus, frequently forming chains in cultures and producing acids freely from carbohydrates. A similar gas-producing type has been described.

**Enterococcus or Micrococcus Ovalis.**—A Gram-positive, oval coccus, usually in pairs or chains. Capsules may be present. Due to delayed cleavage pseudobacillary types may develop on certain media. It is aërobic, facultative anaërobic, non-liquefying, producing acid from glucose and lactose, and coagulating milk.

**B. Putrificus.**—An anaërobic slender, Gram-positive, motile putrefactive bacillus resembling B. tetani in its morphology.

**B. Mesentericus.**—Like B. subtilis, it is essentially a Gram-positive, spore-bearing, aërobic, proteolytic bacillus, that is, forming products of decomposition in protein media when glucose is absent.

**B. Aërogenes (capsulatus).**—See p. 376.

**B. Coli and Allied Types.**—See Chapter XXII.

**Intestinal Flora of Breast-fed Infants.**—The Micrococcus ovalis types preponderate in the duodenum and are most numerous when digestion is in progress. The dominant type in the remainder of the small intestine is B. aërogenes, B. coli being most numerous about the cecal level. B. bifidus finds its optimum in the large intestine. Putrefactive bacteria are uncommon in normal breast-fed infants. Fecal smears show a preponderance of Gram-positive types.

**Intestinal Flora of Artificially-fed Infants.**—The Gram-negative B. coli and B. aërogenes as well as the M. ovalis are relatively increased as compared with the breast-fed infant, while B. bifidus types are diminished, being replaced by colon types and B. acidophilus. Proteolytic action is evidenced in the alkaline reaction of the feces. In general the distribution of the types follows that in breast-fed infants. The character of the flora is susceptible to variation according to the balance of carbohydrate to protein and by changes in the carbohydrate employed.

**Intestinal Flora of Adults.**—The duodenum except during digestion has a low bacterial content. Cocci predominate in the upper small intestine. B. coli types become numerous in the lower small intestine, and together with B. mesenteric types predominate about the cecal level. These and, to a limited extent, the proteolytic anaërobes and certain yeasts constitute the flora of the large intestine. The flora as compared with that of infants is characterized by the absence of essential carbohydrate-fermenting types. The carbohydrate of adult life is mostly starch and the products of its cleavage are probably quickly absorbed, leaving the more slowly cleaved and absorbed protein as available foodstuff for the bacteria. The colon types and the aërobic and anaërobic proteolytic types which can utilize this material therefore dominate the flora.

**Variations in the Bacterial Flora and Their Significance.**—The flora as given for the three types is basically dependent on the diet. Thus, in the nursing infant, the carbohydrate is sufficient in amount to supply the conditions for growth of the essentially fermentative types. These because of their acid production limit to a marked degree the multiplication of other types, especially the proteolytic types. Although in the case of the artificially fed infant the available carbohydrate is less in amount, it is still sufficient to lead to relatively the same result. If, however, the protein proportion of the food be increased, a relative or complete suppression of acid-producing types with an increase of the proteolytic types occurs, approximating the flora of the adult. The activity of B. coli as regards end-products depends also on the availability of carbohydrates. If present in sufficient amounts, lactic acid and other end-products of carbohydrate fermentation are produced. When absent, the end-products of protein cleavage, $H_2S$,$NH_3$, and indol, are produced. It is easy to see how carbohydrate fermentation acts as a check to excessive putrefactive activity, which condition is deleterious to the host. The bacterial flora, as a rule, is little influenced by the ingestion of bacteria in the foods.

When infection occurs, such as with dysentery or cholera, these organisms and others similar to them may dominate the intestinal flora and a specific disease develops. Apart from these well-known types of disease there are other morbid conditions which, although of unknown etiology, are intimately associated with changes of the intestinal flora, even if these changes are not of etiological importance. These can be subdivided as to whether (*a*) the products of bacterial proteolysis, (*b*) the products of carbohydrate fermentation or (*c*) both are at fault. The last is the least understood.

In the first case a condition of auto-intoxication develops with the presence of ethereal sulphates in the urine. In the second the production of abnormal or the excessive production of the products of fermentation as simple hyperacidity or less-known irritative products due to the overactivity of B. aërogenes or B. capsulatus are the probable cause of the symptoms. Diarrheal conditions due to the latter, even though chronic, do not show evidences of toxemia.

On this basis attempts have been made to influence these conditions. Cathartics, intestinal antiseptic and starvation have no appreciable beneficial value. Changes in diet so adapted as to limit or supply the optimum foodstuff for certain types is apparently the most important controlling factor. An increase of carbohydrates leads to a flora dominated by B. acidophilus types. Not all carbohydrates, however, have an equal tendency to bring about this transformation to a dominant fermentative flora. Lactose and dextrin are most effective. (Torrey.) Proteins likewise differ in their effect on the putrefactive flora. Those of milk are less likely to increase this flora than those of meat. Vegetable proteins apparently do not encourage an increase of these types. Metchnikoff's sour-milk therapy was an attempt to modify the flora through the introduction of exogenous lactic acid bacteria and their products. It is very doubtful whether the B. bulgaricus ever becomes acclimated in the intestines. This indicates that the beneficial results of such milk preparations is due to the acids as well as to the character of the foodstuffs and their palatability.

**Types of Sour Milk (Lactic Acid Milks).**—Milk will sour naturally due to lactic acid bacteria which are contaminations of the milk from its surroundings. Buttermilk is a naturally soured product. Metchnikoff advised the use of B. bulgaricus (see below) found in the sour milk of Eastern Europe and in Asia. Many lactic acid-milk preparations are available. To control the appearance of the end-product and to have a uniformly palatable preparation, it is necessary to heat the milk and add a starter. Thus "koumyss" is fermented by lactic acid bacteria and yeasts. "Maadsoun" and "zoolak" by B. bulgaricus and lactic acid producing streptococci and diplococci. Many milk dealers now offer lactic acid milks under various trade names, the starters used being those mentioned in various combinations. B. bulgaricus alone gives too sour a milk.

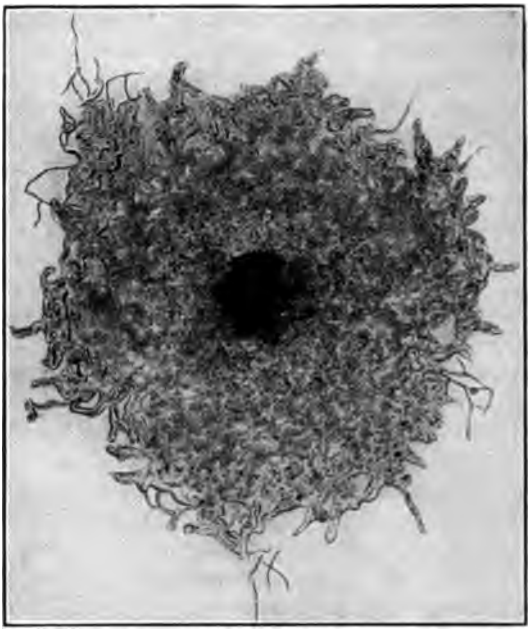

FIG. 130.—B. bulgaricus; seventh day (44°) colony. Whey agar plate. × 50 diameters. (White and Avery.)

**B. Bulgaricus.**—The types included under this name belong to the aciduric group of bacilli. They are non-motile, Gram-positive (except involution forms which are negative) aërobic, facultative anaërobic, non-liquefying bacilli occurring singly or in chains. They require carbohydrates for their growth, growing best on or in milk or whey media, producing large amounts of acids, which, however, do not readily inhibit their growth. On whey agar, they produce characteristic colonies (see Fig. 130). Milk is quickly coagulated. White and Avery have differentiated two groups depending upon the amount of lactic acid produced.

**Other B. Bulgaricus Preparations.**—Other methods of using B. bulgaricus in the form of liquid cultures, dried tablets or oily suspensions are of little value as compared with milk preparations. Their use is based on the assumption that the bacilli will be active in the intestinal tract, which is an assumption of doubtful validity. Furthermore, they do not supply the preformed acid, at least to any extent, as contained in milk preparations. Bendick has found that many of the tablets and probably the oily suspensions as well, contain very few viable organisms;

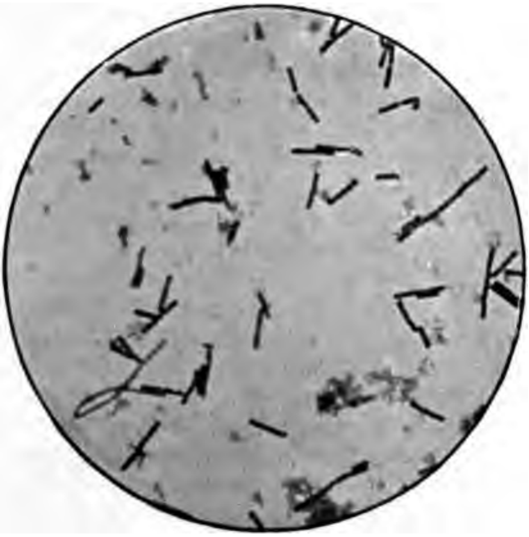

Fig. 131.—B. bulgaricus. × 1000 diameters. (Piffard.)

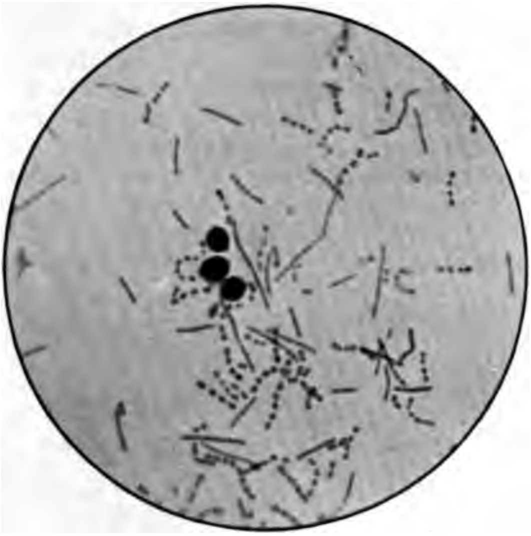

Fig. 132.—"Lactic acid" milk containing B. bulgaricus and a lactose-fermenting streptococcus.

and the bacilli in liquid preparations tend to die out rapidly. At best, this preparation does not compare with sour milk in its bacillary content.

In conditions of toxemia the limitation of proteins with liberal drinking of sour-milk preparations, flooding the intestinal tract with preformed acids is beneficial. If implantation of aciduric types was desired it would seem more logical to feed B. acidophilus which is naturally an intestinal parasite. In the special fermentative types of diarrhea due apparently to an overgrowth by B. capsulatus the liberal use of acid buttermilk and a carbohydrate-free diet will lead to improvement. The same treatment should be applied if B. aërogenes is the apparent causative agent.

**The Influence of Available Foodstuffs in Specific Intestinal Infections.**—In infections due to members of the typhoid-dysentery group or to the cholera vibrio the presence or absence of available carbohydrates may have an important bearing on the products developed by these bacteria and thus on the disease. Each will probably utilize carbohydrate if available, in preference to proteins (protein sparing). There is no evidence that the acids produced from carbohydrate cleavage are any more harmful than those produced by any non-pathogenic fermentative organism. Furthermore, the acid produced would limit their further growth. Not only may the products of protein cleavage be harmful in themselves, but the toxicity of the products of growth, whether a free toxin is produced or not, is enhanced in amount when growth is due to the utilization of protein material.

A free carbohydrate diet, therefore, should be valuable in these diseases not only to spare protein but as an incentive to increased activity of the aciduric types which would aid by limiting multiplication. Although difficulties due to inflammatory conditions of the intestines and other causes may interfere with the efficiency of this mode of treatment, its results in general have been good. Coleman and Schaffer, with the high calory diet in typhoid fever, have not only demonstrated that the nitrogen and weight loss is prevented to a large extent, but that the toxemia is reduced. Liberal feeding of lactose in dysentery has yielded similar results.[1]

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[1] For fuller discussion and bibliography see:
Herter: Bacterial Infections of the Intestinal Tract, New York, 1907.
Kendall: Am. Jour. Med. Sc., 1918, clvi, 157.
Torrey: Jour. Infect. Dis., 1915, xvi, 72.
Torrey: Jour. Med. Res., 1919, xxxix, 415.

## CHAPTER XXII.

## THE COLON-TYPHOID GROUP OF BACILLI.

THERE are a number of varieties of bacilli normally occupying the intestines of man and animals which, because they have similar characteristics and live in the colon, are generally grouped together as colon bacilli. Many of the varieties occurring in animals are culturally like those found in man. These bacilli are only pathogenic under unusual conditions. The specific pathogens, typhoid, paratyphoid, including the types responsible for meat poisoning, dysentery, and paradysentery bacilli, also have among themselves and between them and the colon bacilli resemblances and are often classed together in the group of the colon-typhoid bacilli.

The chief characteristics common to this whole group are: (1) a similar morphology, *i. e.*, short, rather plump non-sporebearing rods with a tendency to thread formation; (2) a Gram-negative staining reaction; (3) similar growths on agar and gelatin; (4) non-liquefaction of gelatin (a few closely related organisms, such as *B. cloacæ*, liquefy gelatin very slowly).

In order to see more clearly the main points of difference among the subdivisions of this great group the following tabulations may be studied.

GROUP OF COLON-TYPHOID BACILLI.

| | | |
|---|---|---|
| Colon group | B. (coli) communis<br>B. (coli) communior<br>B. (acidi) lactici<br>B. aërogenes types | Normal inhabitants of the intestines, under certain conditions become pathogenic. |
| Paratyphoid enteritidis group | B. paratyphosus, A<br>B. paratyphosus, B<br>B. enteritidis<br>(Similar types, intermediates between colon and typhoid have been isolated from water and other sources, probably non-pathogenic.) | Pathogenic for man.[1] |
| B. typhosus | | Pathogenic for man. |
| Dysentery group | B. dysenteriæ (mannite not fermented).<br>B. paradysenteriæ (mannite fermented).<br>Three varieties. | Pathogenic for man, paradysenteriæ usually less so than dysentery type. |
| Alkaligenes group | B. alkaligenes. | Occasionally pathogenic. |

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[1] See page 398 for animal pathogens.

Essential Differential Reactions—Colon-typhoid Group.

| | | |
|---|---|---|
| *Lactose* | *Acid and gas* (colonies, red on Endo and Conradi) | *Glucose—acid and gas—Colon group.* (Russell medium—slant, acid butt, acid and gas.) |
| | *not fermented,* (colonies, colorless on Endo, blue on Conradi) | *Glucose—acid and gas—Paratyphoid-enteritidis group.* (Russell medium—slant, unchanged butt, acid and gas.) |
| | | *Glucose—acid only—B. typhosus—dysentery group.* (Russell medium—slant, unchanged butt, acid only.) |
| | | *Glucose—not fermented—B. alkaligenes.* (Russell medium—slant, unchanged butt, unchanged.) |

Among the non-lactose fermenters motility or its absence is of differential value. The fermentative characteristics of value in differentiating members of the subgroups are given with each subgroup.

**Russell's Medium.**—The reactions as given are based on the fact that the bacilli growing aërobically, that is, on the slant, only utilize the carbohydrate when present in amounts over 0.1 per cent., whereas growing anaërobically stabbed in the butt, they must utilize the carbohydrates for their oxygen supply and therefore ferment the trace of glucose present. By adding 1 per cent. saccharose intermediate types are differentiated.

## THE COLON GROUP.

The first description of an organism of the colon type was by Emmerich (1885), who obtained it from the intestinal discharges of cholera patients. A similar organism was found by Escherich (1886) in the feces of healthy infants. He gave it the name of *Bacterium coli commune.* It has since been demonstrated that closely allied types of bacilli are normal inhabitants of the intestines of most of the lower animals. They are transferred through the feces as manure and sewage to cultivated land, surface waters, etc. During warm weather they may multiply outside of the animal body. Those strains having the chief cultural characteristics of the original strains are classed as colon bacilli, while those differing considerably from it are, while considered in the general group, giving different names, such as paracolon, "atypical coli," etc. These types are especially common in water and their atypical reactions are often due to the suppression of certain characteristics due to unfavorable environment.

The colon group is divided into four main subgroups, *B. communis, B. communior, B. aërogenes,* and *B. (acidi) lactici.* These subgroups have numerous varieties.

The colon group has interest not only because it excites disease at times in man and animals, but also because it is an index of fecal pollution from man or animals. If from man it indicates the possibility of infection with the typhoid or dysentery bacilli. For the significance of the colon group in water see Bacteriology of Water.

**B. (Coli) Communis.**—This organism is taken as the *type* of the group in the following description. Only differentiating points are noted in the other varieties.

**Morphology.**—*B. communis* varies in morphology. The typical form (Fig. 133) is that of short rods with rounded ends, from 0.4μ to 0.7μ in diameter by 1μ to 3μ in length; sometimes, especially when the culture media are not suitable for their growth and in tissues, the rods are so short as to be almost spherical, resembling micrococci in appearance, and, again, they are somewhat oval in form or are seen as threads of 6μ or more in length. The various forms may often be associated in the same culture. The bacilli occur as single cells or as pairs joined end-to-end, rarely as short chains. There is nothing in the morphology of this bacillus sufficiently characteristic for its identification.

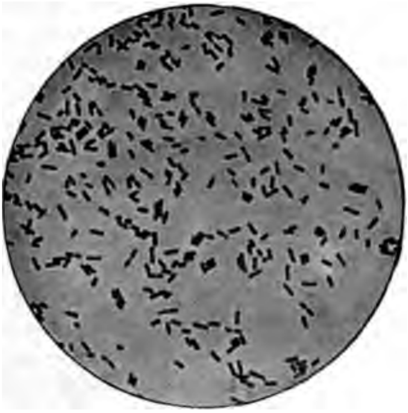


FIG. 133.—Colon bacilli. Twenty-four-hour agar culture. × 1100 diameters.

**Flagella.**—Upon some varieties seven or eight peritrichic flagella have been demonstrated, the non-motile types show none. The flagella are shorter and more delicate than those characteristic of the typhoid bacilli.

**Staining.**—The *B. communis* stains readily with the ordinary anilin colors; it is always decolorized by Gram's method. Under certain conditions the stained bacilli exhibit bipolar granules.

**Biology.**—It is an *aërobic, facultative anaërobic, non-liquefying* non-sporebearing bacillus. It develops best at 37° C., but grows well at 20° C., and slowly at 10° C. It is usually motile, but the movements in some of the cultures are so sluggish that a positive opinion is often difficult. In fresh cultures, frequently only one or two individuals show motility.

**Cultivation.**—The *B. communis* develops well on all the usual culture media. Its growth on them is usually more abundant than that of the typhoid bacillus or the dysentery bacillus, but the difference is not sufficient for a differential diagnosis.

**Gelatin.**—In gelatin plates, colonies are developed in eighteen to thirty-six hours. They resemble greatly the colonies of the typhoid bacillus, except that many of them are somewhat larger and more opaque. (See Figs. 49 to 51, page 136). When located in the depths of the gelatin and examined by a low-power lens they are at first seen to be finely granular, almost homogeneous, and of a pale yellowish to brownish color; later they become larger, denser, darker, and more coarsely granular. In shape they may be round, oval, or whetstone-like. When the gelatin is not firm the margins of many colonies are broken by outgrowths, which are rather characteristic of colon bacilli.

In stab cultures in gelatin the growth usually takes the form of a nail with a flattened head, the surface extension generally reaching out rapidly to the sides of the tube.

**Nutrient Agar.**—In plate cultures: Surface colonies mostly circular, finely granular, and rather opaque. The deep colonies are likely to have protuberances. In streak cultures an abundant soft, white layer is quickly developed, but the growth is not characteristic.

**Bouillon.**—In bouillon the *B. communis* produces diffuse clouding with sedimentation; in some cultures a tendency to pellicle formation on the surface is occasionally seen.

**Potato.**—On potato the growth is rapid and abundant, appearing after twenty-four to thirty-six hours in the incubator as a yellowish brown to dark cream-colored deposit covering the greater part of the surface. There are considerable variations from the typical growth; there may be no visible growth at all, or it may be scanty and of a white color. These variations are often due to variations in the potato.

**Milk.**—Milk is usually coagulated in from one to four days at 37° C. Coagulation is due principally to the production of lactic and acetic acids. Some strains produce acid but coagulation does not occur. It is possible that a lab ferment is partly responsible for the coagulation.

**Chemical Activities.—Behavior Toward Carbohydrates.**—In cultures of *B. communis* many carbohydrates, especially sugars, become fermented with production of acid and gas.

The important fermentation products, both qualitatively and quantitatively, are produced from grape-sugar, probably according to the following reaction:

$$\underset{\text{Grape-sugar.}}{2C_6H_{12}O_6} + \underset{\text{Water.}}{H_2O} = \underset{\text{Lactic acid.}}{2C_3H_6O_3} + \underset{\text{Acetic acid.}}{CH_3COOH} + \underset{\text{Ethyl alcohol.}}{C_2H_5OH} + \underset{\substack{\text{Carbonic}\\\text{acid.}}}{2CO_2} + \underset{\text{Hydrogen.}}{2H_2}$$

There are reasons to think that lactic acid is first produced and that from this other acids and products develop. Under aërobic conditions lactic acid is produced in excess of acetic, while in the absence of oxygen the reverse is likely to be true.

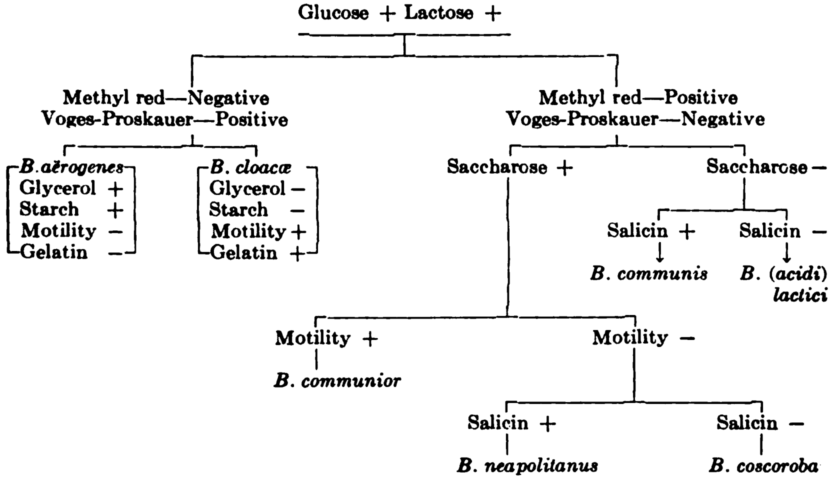
**Gas Production.**—When *B. communis* is grown in a solution of glucose (dextrose), $CO_2$ and $H_2$ are produced, in the proportion of $1CO_2$ to $1H_2$ up to $1CO_2$ to $2H_2$. Anaërobic conditions aid gas formation. Very slight traces of gases other than $H_2$ and $CO_2$ are produced. The amount of gas varies in different varieties; the closed arm of the tube half-filled and the $H_2$ and $CO_2$ in the proportion 2 to 1 is the characteristic type.

The fermentation is not a simple hydrolytic action, but one in which combinations between the C and O atoms are sundered and formed. This is not an oxidation process, but a change through breaking down —that is, a true decomposition. What oxidation takes place is chiefly due to the oxygen liberated from splitting the sugar molecules.

The fermentative reactions of the main subgroups based on the work of Smith[1] and of MacConkey[2] are as follows:

B. (coli) communis—saccharose, negative—dulcit, positive.
B. (coli) communior—saccharose, positive—dulcit, positive.
B. (acidi) lactici—saccharose, negative—dulcit, negative.
B. aërogenes—saccharose, negative—dulcit, negative.

Based on a statistical study of colon varieties obtained from soil, sewage, feces of various animals Levine[3] suggests the following classification.



The last group usually gives a positive Voges-Proskauer reaction (see Bacteriology of Water).

**Effect of B. Communis in Nitrogenous Compounds.—Indol Formation.**— *B. communis* does not liquefy gelatin nor peptonize any albumins. They do, however, break down some of the higher nitrogenous compound into smaller atom groups. The first noted of these compounds was indol, $C_6H_4\left\langle{NH \atop CH}\right\rangle CH$. This is one of the most important products of colon activity, although some varieties lack the ability to produce it. The maximum amount of indol is present about the tenth day. In the intestinal canal in health very little indol appears to be produced by the bacilli. Sulphurated hydrogen is liberated from sugar-free protein substances.

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[1] Jour. Hyg., 1905, v, 333.
[2] Centralbl. f. Bakt., 1895, xviii, 494.
[3] Jour. Bact., 1918, iii, 253.

**Reduction Processes.**—Nitrates are reduced to nitrites and from the latter ammonia and free nitrogen. Litmus and other dyes are also reduced.

**Toxins.**—The bodies of dead bacilli contain pyogenic and other substances, which, injected into the circulation, produce paralysis of the striped muscle fibers, convulsions, coma, and death. Extracts from some cultures produce irritation of the mucous membranes of the large intestines with dysenteric symptoms.

**Growth with Other Bacteria.**—The *B. communis* as well as other members of the colon group act antagonistically to many of the proteolytic bacteria in the intestinal tract, and so inhibit alkaline putrefaction otherwise caused by the latter. In milk the same antagonism exists, probably because of the acidity caused by the colon growth.

**Reaction to High and Low Temperatures.**—*B. communis* is killed at 60° C. in from five to fifteen minutes. Frozen in ice a large proportion die, but some resist for six months. Frozen in liquid air 95 per cent. are killed in two hours.

**Resistance to Drying and Antiseptics.**—Simply drying destroys the majority of organisms dried at any one time, but some bacilli of the number dried may remain alive, especially when held in the texture of threads, for five or six months, or all may die in forty-eight hours.

To most antiseptics they are moderately resistant. They are killed in five to fifteen minutes in a 1 per cent. solution of carbolic acid.

**Effect of Acids.**—*B. communis* grows in a wider range of acids and alkalis than most other bacteria. It develops in from 0.2 to 0.4 per cent. of mineral acids, in from 0.3 to 0.45 per cent. of vegetable acids, and in from 0.1 to 0.2 per cent. of alkalis.

**Effect of Intestinal Juices.**—Gastric juice kills unprotected *B. communis* unless it is too greatly diluted by food. All the members of the typhoid-colon group are more resistant to the gastric juices than most non-sporebearing bacteria. With the food they readily pass from the stomach into the intestines. They grow in bile and in the intestinal juices.

**Pathogenesis.**—**In Lower Animals.**—Intraperitoneal and intravenous inoculation of guinea-pigs and rabbits may produce death, which, when it follows, usually takes place within the first forty-eight hours, accompanied by a decided fall of temperature, the symptoms of enteritis, diarrhea, etc., and finally fibrinopurulent peritonitis.

Subcutaneous inoculation into rabbits is followed usually by abscess formation at the point of inoculation. Dogs and cats are similarly affected.

Cystitis and pyelonephritis may be produced by direct injections into the bladder and ureters, if the urine is artificially suppressed. Angiocholitis and abscess are produced by direct injections into the liver. Osteomyelitis may follow the intravenous injections of cultures in young rabbits.

From experiments on animals it would appear that the explanation of the pathogenesis of the colon bacillus is undoubtedly to be found in the toxic effects of the chemical substances and products of the cells.

**In Man.**—In normal intestines with intact mucous membranes the toxic products formed by *B. coli* are absorbed but little or not at all, and the bacilli themselves are prevented from invading the tissues by the epithelial layer and the bactericidal properties of the body fluids. Possibly there is an acquired immunity to the colon varieties which have long inhabited the intestines.

*B. coli* was at first regarded purely as a saprophyte. Later, because of the postmortem invasion and the great ease of growth of the colon bacillus on ordinary media, the other extreme of attributing too much to it was taken.

The bacilli present in the intestines may, either by an increase in virulence or by a lowered resistance in the person, cause intestinal inflammation or more distant infections. Thus in the case of ulceration in typhoid fever *B. coli* may produce peritonitis. In conditions approaching death they at times pass through the intact mucous lining. The spread of bacilli from the intestines may also cause disease in the gall-bladder or urinary tract. The specific serum reaction in the body is a sign of infection, but great care has to be observed in deciding that it is present, as group agglutinins also occur. Up to the present time it is very difficult to state in any colon infection whether the bacilli were previously present in the intestines or were derived from outside sources through water, food, or direct contact with other cases.

**Intestinal Lesions.**—The lesions present in intestinal inflammation attributed to *B. coli* are those of enteritis; the duodenum and jejunum are found to contain fluid, the spleen is somewhat enlarged, and there are marked hyperemia and ecchymosis of the small intestines, together with swelling of Peyer's patches.

**Virulence of B. Coli from Normal and Diseased Intestines.**—The virulence varies with the culture and the time since its recovery from the intestines. Other things being equal, it is usually more virulent from an intestinal inflammation. From severe diarrhea the colon bacilli in 0.25 c.c. bouillon culture may kill guinea-pigs if given intraperitoneally, while from the healthy bowel 2.5 c.c. are usually required. The difference in man would probably be even greater.

**B. Coli in Sepsis.**—When lesions of the intestinal mucous membranes exist, or in colon cystitis, pyelitis, or cholecystitis, there is frequently just before death a terminal dissemination of the bacilli and consequent septicemia. The colon septicemia is detected by blood cultures. At times very few bacilli are found, and then the blood infection may be less important than the local one. Cases occurring in typhoid and cholera have been observed, especially in relapses in typhoid. In very young infants a malignant septicemia with tendency to hemorrhages may be due to *B. coli*. In a few cases in which *B. coli* but no typhoid bacilli were present the course of the disease has been similar to typhoid fever. An epidemic probably due to colon infection of water has been noted.

Infections through food and water are usually caused by other closely allied bacilli not belonging to the colon group.

**B. Coli in Diarrhea.**—In diarrhea we find increased peristalsis, less absorption of foodstuff, increased and changed intestinal secretions. Tissier observed that under treatment with cathartics the colon varieties increased, while the anaerobic forms are inhibited. In diarrhea, therefore, we should expect favorable conditions for multiplication with the inhibiting causes lessened. This makes one question the importance of the significance of numerous epidemics which have been reported of acute diarrhea in children from one to five years of age in which almost pure cultures of colon bacilli have been found. The symptoms in such epidemics begin with high fever which often rapidly falls, and frequent stools, only watery or containing mucus and streaks of blood. These symptoms may quickly abate or go on to a toxic state characterized by heart weakness and drowsiness. This may lead to lung complications or death.

**B. Coli in Peritonitis.**—Here the lesions must be considered as being due to mixed infection. Not only perforation of the intestines in man, but injury to the intestinal walls, allows colon infection of the peritoneum to take place. At first most of these cases were believed to be a pure colon infection, but now it is known that this idea came largely from the overgrowth of colon bacilli in the cultures. More careful investigations, through cultures and smears, have demonstrated the fact that streptococci, and less frequently staphylococci and pneumococci, are also usually present in peritonitis arising from intestinal sources. The colon bacilli found even in the same case commonly comprise many varieties.

**B. Coli in Inflammation of the Bile Tract.**—The normal healthy gall-bladder is usually sterile. This is true in spite of the fact that bile is apparently a good culture medium for the colon group. Ligation of the neck of the gall-bladder usually causes a colon infection to take place within twenty-four hours. Obstruction of the bile-duct through various causes is fairly common in man. The gall-bladder then becomes infected, and following the inflammation of the mucous membranes there is often the formation of gall-stones. Some cases of jaundice are believed to be due to colon inflammation of the gall-ducts. Atypical varieties of *B. coli* are frequently isolated from gall-bladder infections.

**Inflammation of the Pancreas.**—Welch was the first to record a case of pancreatitis with multiple fat necroses due to *B. coli* infection. A few more cases have since been reported due to members of the colon group, either alone or in conjunction with the pyogenic cocci.

**Inflammation of the Urinary Tract.**—As far back as 1879 Bouchard noted cystitis due to bacilli of the colon group. When cystitis is established the bacterial infection frequently spreads to the pelvis of the kidneys, causing a pyelitis or suppurative nephritis. In most cases of chronic cystitis the ureters and pelves of the kidneys become involved; any malformation of the ureters aids the process. From the pelvis the bacteria push up into the urinary tubules and excite inflammation and multiple abscesses. Colon infection of the different parts of the urinary tract may occur at any age, from infancy upward. Instead of starting in the bladder it may begin in the kidney itself, the colon bacilli coming from the blood or peritoneum. In many of these cases the bacilli isolated from the urine are agglutinated in high dilutions of the blood from the patient.

Although other bacteria—the pyogenic cocci, the proteus, the typhoid bacillus, etc.—may excite cystitis, still in 90 per cent. of all cases some of the colon group are found, and this percentage is even higher in young children. The clinical picture of colon infection is very variable. The lightest cases progress under the guise of a bacteriuria. The urine is passed a little more frequently and shows a fine granular cloudiness. The reaction is acid. The cell elements are but little increased. There is an excess of mucus. Albumin is absent or present in only a trace. The condition may last for weeks or months and then spontaneously disappear or grow worse. With a somewhat more severe infection there is painful urination, perhaps tenesmus, increase of pus cells and slight fever. In a conical glass a sediment of pus cells forms at the bottom, and clear urine remains above. If the infection passes to the kidney colicky pain and tenderness over the region of the kidneys is usually present. The most important symptom of pyelitis is an irregular intermittent fever resembling malaria. Septicemia may finally result.

In most of these cases the microscopic examination is sufficient to make a probable diagnosis, since the bacteria are so abundant. In the urine they appear as diplobacilli, or partly in short, almost coccus, forms, partly in long threads. As a rule motility is absent.

The characteristics of the urine itself have much to do with the probability of infection; the more acid urines being less likely to afford a proper soil for growth. Some urines are bactericidal even when they are neutral. The substances producing this condition are not known. The colon bacilli in the urine produce no appreciable effect on the reaction, but give up some of their toxins, which upon absorption cause the deleterious local and general effects. The serum of the patient usually agglutinates the cultures from the urine in 1 to 20 or 1 to 50 dilutions, but this property is sometimes absent, especially in light cases.

In all cases in addition to the introduction of the colon bacillus a predisposing condition must be present, such as more or less marked retention of urine by an enlarged prostate or stricture any unhealthy state of the mucous membrane or general depression of vitality.

**B. Coli as Pus Former.**—Members of this group are frequently the cause of abscesses in the region of the rectum, urethra, and kidney. They rarely produce pus in other locations.

**B. Coli in Inflammation Not Previously Mentioned.**—Bronchopneumonia, lobar pneumonia, and pleurisy have occasionally been caused by colon bacilli, probably from blood sources. Not a few cases of meningitis and spinal meningitis in infants, follow localized *B. coli* infections. The symptoms are not well developed as a rule. Some cases of endocarditis and of conjunctivitis have also been noted.

**Immunity.**—Natural infection in man, or inoculation into animals, is followed by the production of antibodies; agglutinins, precipitins, bactericidal substances, and opsonins being produced. The attempts to separate the members of this group by agglutination have shown the great dissimilarity of the different members in their immune reactions, although group reactions are marked. Natural agglutinins for this group are commonly present in the serum of man and animals. (See Agglutination.)

**Vaccine Therapy.**—See Part III.

**Methods of Isolation.**—They may be isolated from lesions on ordinary media, in bile media or on Endo or Conradi plates. The latter are used in isolation from feces or other mixed material. Blood cultures may be made in bile or broth. For the examination of water for members of the colon group, see Bacteriology of Water.

**Bacillus Aërogenes and Allied Encapsulated Bacilli.**—The members of this group differ from the members of the colon group, already described, in producing a viscid or mucoid growth, and in smears a capsule is commonly demonstrable. For this reason the group is spoken of by some as the capsulatus group, and the aërogenes types described as allied varieties. There are wide variations in the group, not only in the degree of capsule production but in the ability to ferment different carbohydrates. In the absence of an accepted biological classification of types, the varieties are best considered according to their source. For this reason the term *B. aërogenes* is collectively used for the types normally found in feces, milk, etc.

**B. Aërogenes.**—Normally present in feces and also found in sewage and water. It is constantly present in milk and is one of the chief causes of the souring of milk and cream.

Morphologically it is variable in length and capsule production. In cultures the growth is abundant and viscid or mucoid in variable degree. It usually ferments the various carbohydrates more vigorously than other colon types, the closed arm of the fermentation tube being usually full or nearly full of gas. Indol production is variable. *B. aërogenes* is much more resistant to acids and other deleterious substances than the other colon types.

*Pathogenesis.*—Probably slight, although it has been isolated from infections of the urinary tract, peritoneum and liver and gall-bladder. In some of the cases reported there is a possibility that the infection was really due to other of the colon types. In the absence of reliable methods of differentiation there is always a question whether the more serious infections are not due to the more virulent bacillus of Friedländer.

**Bacillus Pneumoniæ, Friedländer (B. Mucosus Capsulatus).**—This bacillus was first described by Friedländer in 1882. He confused this bacillus with the pneumococcus, then undescribed, and regarded it as the causative agent in lobar pneumonia.

*Morphology.*—It varies from coccoid forms to longer bacilli; they may be single, in pairs, or in short chains. They are surrounded by a wide capsule which is easily demonstrable not only in the material directly from the lesions but also in smears from cultures.

*Biology.*—It grows freely on ordinary media. On agar the colonies are characteristically mucoid, with a tendency to confluence. Indol is not produced.

*Pathogenesis.*—It is pathogenic for mice and guinea-pigs in variable degree. Injection intraperitoneally or into the lung is followed by peritonitis or local hepatization with septicemia. Rabbits are killed by some strains after intraperitoneal or intravenous injection; subcutaneous injection, however, commonly causes only a local lesion.

In man it causes both broncho- and lobar pneumonia. This type of pneumonia is relatively infrequent and is characterized by its very high mortality. The bacilli are present in the sputum, as a rule, in almost pure culture. It is commonly present in the upper respiratory tract and the accessory sinuses, and may cause inflammation. By extension, infection of the ear occurs, which may be complicated by meningitis. The pleura or the pericardium may be infected as a complication of pneumonia. A septicemia may develop as a complication of pneumonia or infection elsewhere. Inflammations of the eye are occasionally due to this organism.

**B. Ozena.**—This organism is culturally indistinguishable from the preceding. It receives its name from its constant presence in ozena or fetid rhinitis. Abel and others consider it the causative agent in this disease, basing their opinion on its constant presence.

**Bacillus of Rhinoscleroma.**—This bacillus differs from the *Bacillus pneumoniæ* only in its weaker fermentative properties. Its name comes from its frequent presence in rhinoscleroma. Its etiological relationship to this disease is not established. (See Fig. 13.)

**Immunity and Serum Reactions of the Capsulatus Types.**—Active immunity cannot, as a rule, be produced in animals by the injection of killed cultures or their products. Careful injections of animals, however, lead to the production of agglutinins, precipitins, and complement-fixing antibodies. The attempt to differentiate the various types by means of serum reactions has as yet led to no uniform results.

In common with other encapsulated bacteria they are not easily affected by sera. Porges found that the absence of agglutination could be attributed to the presence of the capsule and devised a method of destroying the capsule by first treating the bacterial suspension with acids and heat and then neutralizing with alkali.

**Vaccine Therapy.**—See Part III.

**Other Bacilli Allied to the Colon Group.**—There are various types, of which *B. cloacæ* is an example, which resemble the colon bacillus in many respects but liquefy gelatin in varying degree, some very slightly and only after prolonged growth.

# CHAPTER XXIII.

# THE TYPHOID BACILLUS.

This organism was first observed by Eberth, in 1880, in the spleen and diseased areas of the intestine in typhoid cadavers, but was not obtained in pure culture or its principal biological features described until the researches of Gaffky in 1884. The absolute identification of the bacillus only became possible with the increase of our knowledge concerning the specific immune substances. Its etiological relationship to typhoid fever has been particularly difficult to demonstrate, for although pathogenic for many animals when subcutaneously or intravenously inoculated, it was impossible to produce infection in the natural way. More recently infection of the higher apes has been produced. Nevertheless, the specific reactions of the blood serum of typhoid patients, the constant presence of the *Bacillus typhosus* in the intestines and some of the organs of the typhoid cadavers, the very frequent isolation of this bacillus from the roseola, spleen, blood, and excretions of the sick during life, the absence of the bacilli in healthy persons, unless they have at some time been directly exposed to, or are convalescent from, typhoid infection, all these have demonstrated scientifically that this bacillus is the chief etiological factor in the production of the great majority of cases designated as typhoid fever.

**Morphology and Staining.**—Typhoid bacilli are short, rather plump rods of about 1$\mu$ to 3$\mu$ in length by 0.5$\mu$ to 0.8$\mu$ in diameter, having rounded ends, and often growing into long threads. They are longer and somewhat more slender in form than most of the members of the colon group of bacilli (Figs. 134 and 135).

The typhoid bacilli *stain* with the ordinary anilin colors, but a little less intensely than do most other bacteria. Like the bacilli of the colon and paratyphoid groups, they are decolorized by Gram's method. Bipolar staining is sometimes marked.

**Biology.**—The typhoid bacillus is a motile, aërobic, facultative anaërobic, non-liquefying bacillus. It develops best at 37° C.; above 40° and below 30° growth is retarded; at 20° it is still moderate; below 10° it almost ceases. It grows slightly more abundantly in the presence of oxygen. It does not form spores.

**Resistance.**—When a number of typhoid bacilli are dried most of them die within a few hours, the remainder gradually dying during the next few weeks. A few frequently remain alive for months. In their resistance to heat and cold they behave like the average non-sporebearing bacilli. With rare exceptions they are killed by heating to 60° C. for one minute.

**Motility.**—Typhoid bacilli, when living under favorable conditions, are very actively motile, the smaller ones having often an undulating motion, while the larger rods move about rapidly. In different cultures, however, the degree of motility varies.

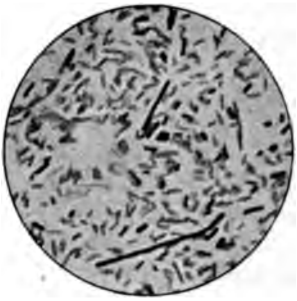

FIG. 134.—Typhoid bacilli from nutrient agar. × 1100 diameters.

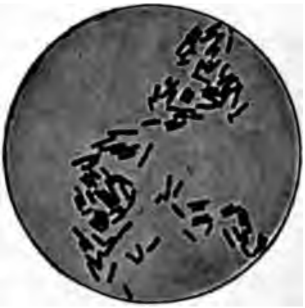

FIG. 135.—Typhoid bacilli from nutrient gelatin. × 1100 diameters.

**Flagella.**—These are often numerous and spring from the sides as well as the ends of the bacilli, but many short rods have but a single terminal flagellum (Figs. 136 and 137; see also Plate III).

**Cultivation.**—Its growth on most sugar-free culture media is quite similar to that of the *Bacillus coli*, but it is somewhat slower and not quite so luxuriant.

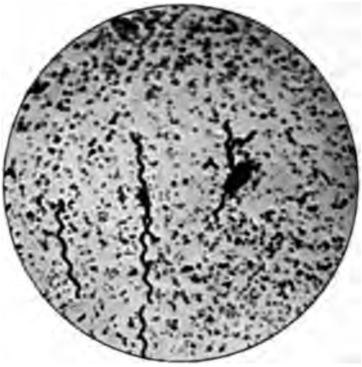

FIG. 136.—Flagella, heavily stained, attached to bacilli. (Van Ermengen's method.)

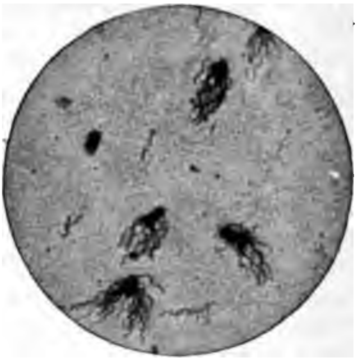

FIG. 137.—Typhoid bacillus with faintly stained flagella. (Löffler's method.)

**Growth on Gelatin Plates** (Fig. 138).—The colonies growing deep down in this plate medium have nothing in their appearance to distinguish them from submerged colonies of the colon group; they are finely granular, with a sharp margin and a yellowish-brown color. The superficial colonies, however, particularly when young, are often quite characteristic; they are transparent bluish white in color, with an irregular outline, not unlike a grape leaf in shape. Slightly magnified they appear homogeneous in structure, but marked by a delicate network of furrows. Surface colonies from some varieties of colon bacilli give a similar picture.

In *stick cultures* in gelatin the growth is mostly on the surface, appearing as a thin, scalloped extension, which gradually reaches out to the sides of the tube. In the track of the needle there is but a limited growth, which may be granular or uniform in structure, and of a yellowish-brown color.

**Growth in Bouillon.**—This medium is uniformly clouded by the typhoid bacillus, but the clouding is not so intense as with the colon bacillus. When the bouillon is somewhat alkaline a delicate pellicle is sometimes formed on the surface after eighteen to twenty-four hours' growth.

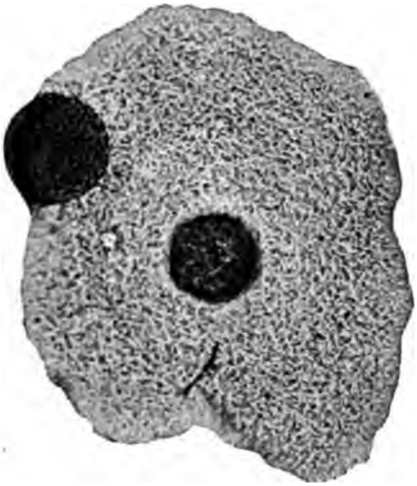

FIG. 138.—A superficial colony and a deep colony of typhoid bacilli in gelatin. × 20 diameters.

**Growth on Agar.**—The streak cultures on agar are not distinctive; a transparent, filiform, grayish streak is formed.

**Growth on Potato.**—The growth on this medium was formerly of great importance in identification, but now other media, giving more specific characteristics, have been discovered. When characteristic, the growth is almost invisible but luxuriant, usually covering the surface of the medium. Again, the growth may be quite heavy and colored yellowish brown, and with a greenish halo, when it is very similar to the growth of the colon bacillus. These differences of growth on potato appear to be chiefly due to variations in the substance of the potato, especially in its reaction. For the characteristic growth the potato should be slightly acid. A new lot of potato should always be tested with a typical typhoid bacillus as a control.

**Indol Reaction.**—It does not produce even a trace of indol in peptone-water solution. This test was proposed by Kitasato for differentiating the typhoid bacillus from other similar bacilli such as those of the colon group, which, as a rule, give the indol reaction.

The typhoid bacillus, like the colon bacillus, produces alkaline substances from peptone.

**Neutral Red.**—In stick cultures in glucose agar or glucose broth cultures with neutral red as indicator the typhoid bacillus produces no change, while the colon bacillus decolorizes the medium and produces gas.

**Effect of Inhibiting Substances in Culture Fluids.**—The typhoid bacillus is inhibited by weaker solutions of formaldehyde, carbolic acid, and other disinfectants than is the colon bacillus. Most typhoid-like bacilli resemble the typhoid bacillus in this respect. Some substances, such as brilliant green, inhibit the colon bacillus more.

**Action on Different Sugars.**—The essential fermentative difference between the typhoid bacillus and paratyphoid colon group are given in Chapter XXII. Acid is produced in mannit, maltose and xylose media. Dulcit is acted upon, as a rule, after prolonged incubation.

While the typhoid bacillus does not produce gas from dextrose galactose, and levulose, it does produce acid from these substances.

**Milk.**—The typhoid bacillus does not cause coagulation when grown in milk. A very slight acidity is evidenced in litmus milk from its action on traces of fermentable substances other than lactose.

**Production of Disease in Animals.**—It is impossible, with the exception of the anthropoid apes, to produce a disease like typhoid fever in animals. Metchnikoff and Besredka fed apes with typhoid bacilli by adding fecal material to their food. After eight days' incubation, fever, diarrhea and invasion of the blood stream by the typhoid bacilli, developed. The general clinical picture resembled that of typhoid fever. Some of the animals died. Injection of other animals may produce sickness due to the toxemia produced by the substances in the bodies of the bacilli injected. Typhoid bacilli, freshly obtained from typhoid cases and introduced subcutaneously in animals, rapidly die. In the peritoneal cavity they may increase, causing a fatal peritonitis with toxic poisoning. By accustoming bacilli to the animal body a certain degree of increased virulence for the animal can be obtained, so that smaller amounts of culture may prove fatal. In rabbits a septicemia can be produced by intravenous inoculation. Localization in the gall-bladder follows in some and a "carrier" state similar to that found in man results. Direct injection into the gall-bladder also results in the development of the carrier state.

**Distribution of Bacilli in the Human Subject. Toxic Effects.**—Typhoid fever during its early stages, at least, is accompanied by a bacteriemia. The bacilli thus pass to all parts of the body and become localized in certain tissues, such as the bone-marrow, lymphatic tissues and spleen, liver and kidneys. Wherever found in the tissues the typhoid bacilli are usually observed to be arranged in groups or foci; only occasionally are they found singly. These foci are formed during life, as is proved by the degenerative changes often seen about them; but it is possible that the bacilli may also multiply somewhat after death.

**Important Primary Characteristic Lesions in Man.**—The lesions of the intestines which are most pronounced in the lower part of the ileum consist of an inflammatory enlargement of the solitary and agminated lymph nodules. Necrosis with ulceration frequently follows the hyperplasia in the more severe cases. In the severest cases the ulceration and sloughing may involve the muscular and peritoneal coats and perforation may occur. Peritonitis and death usually follow. In rare cases the perforation is closed by adhesions.

The minute changes are a hyperplasia of normal elements of the lymphatic tissue, namely, the lymph cells and the endothelium of the trabeculæ and sinuses. In severer forms necrotic changes are apt to intervene. These changes are attributed to the toxic substances formed by the typhoid bacilli, but may be directly brought about by the occlusion of the nutritive blood-vessels, as pointed out by Mallory.

The mesenteric lymph nodes undergo changes similar to those in the ileum. The spleen is enlarged because of congestion and hyperplasia. The liver and, to a less extent, the kidneys are apt to show foci of cell proliferation.

In typhoid fever, as in other infectious diseases, toxic poisoning may be manifested by disturbances in the circulatory, respiratory, and heat-regulating mechanism as well as by manifest lesions. In a few cases the intestinal lesions are absent. Some of the inflammatory complications which occur in typhoid fever are due to the growth of the bacillus in excessive numbers in unusual places in the body; but many of them are due to a secondary infection with other bacteria, especially the pyogenic cocci and bacilli of the colon group.

**Unusual Location of Typhoid Lesions Occurring as Complications of Typhoid Fever.**—Cases of sacculated and general peritonitis, abscess of the liver and spleen, subphrenic abscess, osteomyelitis, periostitis, and inflammatory processes of other kinds have been reported as being due to the typhoid bacillus. In certain cases of typhoid pneumonia, serous pleurisy, empyema, and inflammations of the brain and spinal cord or their membranes, typhoid bacilli exclusively have occurred. The. inflammation produced may or may not be accompanied by the formation of pus. There are indeed a number of cases now on record in which the typhoid bacillus has played the part of *pus producer.*

**The Importance of Mixed Infection.**—Frequently when complications occur in typhoid fever they are due to secondary or *mixed infection* with the staphylococcus, pneumococcus, streptococcus, pyocyaneus, and colon bacillus. Frequently these bacteria are found side by side with typhoid bacilli; in such cases it is difficult to say which was the primary and which was the secondary infection.

**Primary Infection of Liver and Gall-bladder.**—We find an appreciable number of chronic typhoid carriers with no history of having had typhoid. Although the symptoms may have been so slight as to have been overlooked, it is probable that bacilli may escape from the intestine into the portal circulation and thus infect the liver and gall-bladder (see Normal Carriers).

**Elimination of Typhoid Bacilli from Body.**—Not infrequently typhoid bacilli are found in the secretions. They are present in the urine in about 25 to 50 per cent. of the cases during some part of the disease. Slight pathological lesions in the kidneys almost always occur in typhoid fever, but severe lesions also sometimes occur. In some cases the urine has very many typhoid bacilli.

In cases of pneumonia due to the typhoid bacillus it is abundantly present in the sputum, and care should be taken to disinfect the expectoration of typhoid patients. In typhoid fever the bacilli are almost always present in the gall-bladder. The bacilli are usually eliminated by the feces, being derived from the bile and from the ulcerated portions of the intestines; their growth within the intestinal contents is, with few exceptions, not extensive. They may be excreted, however, very early in the disease, and have been found in the stools even during the incubation period.

Not only the great majority of cases examined bacteriologically and pathologically, but the epidemiological history of the disease, proves that the chief mode of invasion of the typhoid bacillus is by way of the mouth and stomach. The infective material is discharged principally by means of the excretions and secretions of the sick—namely, by the feces, the urine, and occasionally by the sputum.

**Typhoid Carriers.**—The bacilli usually disappear from the body during the first week or two of convalescence. About 1 to 5 per cent. continue to excrete typhoid bacilli for years, perhaps for life. Petruschky, in 1898, reported that typhoid bacilli sometimes remained in the urine of typhoid convalescents for months. Cushing soon after observed a case who had had typhoid fever five years before. In 1902 Frosch, and a little later Conradi and Drigalski, reported persons who passed typhoid-infected feces months after recovery from typhoid fever. Some bacilli carriers did not know either that they had had typhoid fever or been in contact with it, and others knew only that they had been in contact with it.

In Washington, during the typhoid season, a series of one thousand examinations showed that 0.3 per cent. of persons with no known exposure, and who had not had typhoid were excreting typhoid bacilli. These cases may be called "healthy or normal carriers" in contradistinction to "convalescent carriers." Lentz, in 1905, found out of a large number of examinations that about 4 per cent. of persons convalescent from typhoid fever were typhoid carriers. In our laboratory we have found 6 in 140 institution convalescents. The focus of infection is either the gall-bladder or bile ducts. The majority of carriers are women.

A remarkable case of a cook ("Typhoid Mary") discovered by Soper, has been under our care for twelve years. A visitor of the family in which this woman was cook developed typhoid fever some ten days after entering the household. This was in 1901. The cook had been with the family three years and it is difficult to judge which infected the other. The cook went to another family. One month later the laundress in this family was taken ill.

In 1902 the cook obtained a new place. Two weeks after arrival the laundress was taken ill with typhoid fever; in a week a second case developed and soon seven members of the household were sick.

In 1904 the cook went to a home in Long Island. There were 4 in the family as well as 7 servants. Within three weeks after arrival 4 servants were attacked.

In 1906 the cook went to another family. Between August 27 and September 3, 6 out of its 11 inmates were attacked with typhoid. At this time the cook was first suspected. She entered another family on September 21. On October 5 the laundress developed typhoid fever.

In 1907 she entered a family in New York City, and two months after her arrival 2 cases developed, 1 of which proved fatal.

The cook was removed to the hospital March 19, 1907. Cultures taken every few days showed bacilli off and on for three years. Sometimes the stools contained enormous numbers of typhoid bacilli and again for days none would be found. She was released on parole in

1910, promising to report to the Health Department and not to engage in cooking. She broke her parole and disappeared. In 1915, in an epidemic of typhoid at a maternity hospital, a total of 25 cases developed. Investigation showed that food infection was the cause and the cook was identified as "Typhoid Mary." During the period of disappearance she infected a friend and was the cause of several cases in a small private sanatorium and 27 in a maternity hospital. Since then she has been again isolated. She is known to have been the cause of at least 50 cases of typhoid fever. We recently traced some hundreds of cases of typhoid fever to a milk supply produced at a farm, looked after by a typhoid carrier who had typhoid fever forty-seven years ago.

**Treatment of Typhoid Carriers.**—Medicinal treatment or surgery seems so far to have yielded only slight results. Urotropin in very large amounts is reported to have cured one case, in which operation alone had failed. Removal of the gall-bladder cannot be relied upon, as the bacilli may be present in the bile ducts. Meader reports some success with long-continued treatment by injections of killed cultures. Our results have been slightly favorable.

**Duration of Life in Water, Feces, Oysters, etc.**—It is of importance to know for what length of time the typhoid bacillus is capable of living outside of the body; but, unfortunately, owing to the great difficulties in proving the presence of this organism in natural conditions, our knowledge on this point is still incomplete. In feces, in privies, on the ground, etc., the length of life of the typhoid bacilli is very variable, depending on the composition of the feces and the soil, and on the varieties of bacteria present; sometimes they live but a few hours usually a day, exceptionally for very long periods. Thus, according to Levy and Kayser, in winter typhoid bacilli may remain alive in feces for five months. Foote says that they can be found in living oysters for a month at a time, but in numerous experiments we have not been able to find them after five days. Their life in privies and in water is usually short, often not over forty-eight hours and usually not over a week. The less the general contamination of the water, the longer the bacilli are apt to live. The life of the typhoid bacillus varies according to the abundance and varieties of the bacteria associated with it, and according to the presence or absence of such injurious influences as deleterious chemicals, high temperature, light, etc., to which it is known to be sensitive. In ice typhoid bacilli rapidly die, none probably ever live as long as six months (see p. 392).

**Methods of Communication.**—The bacilli may reach the mouth by means of infected fingers or articles of various kinds, or by the ingestion of infected food, milk, water, etc., or more obscure ways, such as the eating of raw oysters and clams or the contamination of food by flies. Of great importance, though gradually lessening, however, is the production of infection by contaminated drinking water or milk. In a very large number of cases indirect proof of this mode of infection has been afforded by finding that the water had been contamined with urine or feces from a case of typhoid. In a very few instances the proof has been direct—namely, by finding typhoid bacilli in the water. Examples of infection from water and milk have frequently come under our direct observation. The following instances may be cited: A large force of workmen obtained their drinking water from a well near where they were working. Typhoid fever broke out and continued to spread until the well was filled up. Investigation showed that some of the sick, in the early stages of their disease, repeatedly infected the soil with their urine and feces at a point which drained into the well. Another example occurred in which typhoid fever broke out along the course of a creek after a spring freshet. It was found that, far up near the source of the creek, typhoid feces had been thrown on one of its banks and had then been washed into the stream.

The epidemic at Scranton, Pa., during the winter of 1907 was most interesting. A little over 1 per cent. of the inhabitants were attacked. No pollution of the water with typhoid feces or urine could be discovered, although typhoid bacilli were isolated from the water of a small intercepting reservoir by Dr. Fox. This was only accomplished by using large quantities of water. The bacillus isolated was identical by all known tests with the typhoid cultures from cases of typhoid fever. A stream entering the reservoir was exposed to pollution by men working on a nearby railroad.

In water-borne outbreaks of typhoid fever it is not infrequent to have outbreaks of diarrhea preceding or accompanying the development of the typhoid fever. The number of cases of diarrhea may be many times greater than the number of typhoid cases. A sudden outbreak of diarrhea therefore may mean contamination of a water supply and should be considered as possible indication of an oncoming typhoid outbreak.

An instance of milk infection secondary to water infection occurred in the case of a milk dealer whose son came home suffering from typhoid fever. The feces were thrown into a small stream which ran into a pond in which the milk cans were washed. A very alarming epidemic of typhoid developed, which was confined to the houses and asylums supplied with this milk. Milk-borne epidemics are most commonly due to infection of the milk by carriers. During the Spanish-American War not only water infection, but food infection was noticed, as in the case of a regiment in which certain companies were badly infected, while others nearly escaped. Each company had its separate kitchen and food supply, and much of the infection could be traced to the food, the contamination coming partly through the flies. A small number of cases have been traced to oysters. *Contact infection* is responsible for many cases. Even during outbreaks due to various causes, contact infection plays a large role in the development of secondary cases. These cases develop either by contact with known typhoid cases or through convalescent or healthy carriers in the households. The early undiagnosed cases may have typhoid bacilli in their stools and act as a source of contagion; likewise short, mild cases develop which are never diagnosed. For these reasons "typhoid precautions" should not await positive diagnosis, but should be instituted on the slightest suspicion.

**Individual Susceptibility.**—In this, as in all infectious diseases, *individual susceptibility* plays an important role in the production of infection. Where exposure is associated with the ingestion of few bacilli, the majority of persons escape infection. In many individuals there probably is some disturbance of the digestion, excesses in drinking, etc., or a general weakening of the power of resistance of the individual, caused by bad food, exposure to heat, overexertion, etc., as occurs with soldiers and prisoners, for example, to bring about the conditions suitable for the production of typhoid fever, when the dose of bacilli is small. With ingestion of larger numbers of bacilli and especially with repeated exposure, 50 per cent. or more of the exposed succumb to infection.

**Immunization.**—After recovery from typhoid fever a considerable immunity is present which lasts for years. This is not absolute, as reports indicate that about 2 per cent. of those having typhoid fever have a second attack, which is usually a mild one. Some of these apparent recurrences may have been paratyphoid fever. Specific *immunization* against experimental typhoid infection has been produced in animals by the usual method of injecting at first small quantities of the living or dead typhoid bacilli and gradually increasing the dose. The blood serum of animals thus immunized has been found to be highly bactericidal and to possess some protective power against toxic extracts of the bacilli. It is also rich in agglutinins, precipitins, and opsonins. These characteristics have also been observed in the blood serum of persons who are convalescent from typhoid fever. The attempt has been made to employ the typhoid serum for the cure of typhoid fever in man, but, although a number of individual observers have reported good results with one or another of the serums, most consider that little or no good is derived from serum and it is not at present used.

**Vaccine and Serum.**—See Part III.

**Diagnosis by Means of the Widal or Agglutination Reaction.**—The chief practical application of our knowledge of the specific substances developed in the blood of persons sick with typhoid fever has been as an aid to diagnosis.

**Gruber-Widal Test.**—The first application of the use of serum for the early diagnosis of typhoid fever on an extensive scale was made by Widal, and reported with great fulness and detail in a communication published in June, 1896. Since then the serum test for the diagnosis of typhoid fever has come into general use in bacteriological laboratories in all parts of the world, and though the extravagant expectations raised at the time when Widal first announced his method of applying this test have not been entirely fulfilled, it has, nevertheless, proved to be of great assistance in the diagnosis of obscure cases of the disease, and is now one of the recognized tests for the differentiation of the typhoid bacillus.

It should also be mentioned that to Wyatt Johnson, of Montreal, belongs the credit of introducing its use into municipal laboratories, suggesting that dried blood should be employed in place of blood serum.

**Technic of Test.**—See chapter on Agglutination.

**Advantages and Disadvantages of Serum, Dried Blood, and Fluid Blood for the Serum Test.**—The dried blood is easily and quickly obtained, and does not deteriorate or become contaminated by bacterial growth. It is readily transported, and seems to be of nearly equal strength as the serum in its agglutinating properties. It must in use, however, be diluted with at least five times its original bulk with water, otherwise it is too viscid to be properly employed. The amount of dilution can be determined roughly by the color of the resulting mixture. Serum, on the other hand, can be used in any dilution desired, from a mixture which contains equal parts of serum and broth culture to any dilution desired; and this can be exactly measured by a graduated pipette, or, roughly, by a measured platinum loop. The disadvantages in the use of serum are entirely due to the slight difficulty in collecting and transporting it. If the serum is obtained from blood after clotting has occurred a greater quantity of blood must be drawn than is necessary when the dried-blood method is used. For scientific investigations and for accurate results, particularly in obscure cases, the use of serum is to be preferred to dried blood. Practically, however, the results are nearly as good for diagnostic purposes from the dried blood as from the serum.

**The Cultures to be Employed.**—The culture employed must be suitable (see chapter on Agglutination). A strain known as Mt. Sinai is now employed at this laboratory.

**Dilution of the Blood Serum to be Employed and Time Required for the Development of Reaction.**—The serum test, as has been pointed out, is quantitative and not qualitative, that is, the diagnostic reaction must be in dilutions higher than occur because of the presence of normal or group agglutinins. It is most important to remember that it is purely a matter of experience to determine in any type of infection what agglutinating strength of a serum is of diagnostic value.

The results obtained in the Health Department Laboratories, as well as elsewhere, have shown that in a certain proportion of cases not typhoid fever a slow reaction occurs in a 1 to 10 dilution of serum or blood; but very rarely does a complete reaction occur in this dilution within *fifteen minutes*. When dried blood is used the slight tendency of non-typhoid blood in 1 to 10 dilution to produce agglutination is increased by the presence of the fibrinous clumps, and perhaps by other substances derived from the disintegrated blood cells.

From many cases examined it has been found that in dilutions of 1 to 20 a quick reaction is almost never produced in any febrile disease other than that due to typhoid or paratyphoid bacillus infection. In typhoid fever such a distinct reaction often occurs with dilutions of 1 to 100 or more. In some cases of paratyphoid infection a prompt reaction in 1 to 20 dilutions is obtained, but if this is so, it is not a serious drawback. As has been noted under agglutination titration of the agglutinin content may be employed to make a presumptive differential diagnosis between typhoid and paratyphoid fever. Typhoid-bacillus carriers with the exception of transient contact or normal carriers, commonly have specific agglutinins in their blood whether they previously have had typhoid fever or not.

The mode of procedure as now employed is as follows: With serum, one part of a 1 to 10 dilution is added to one of the bouillon cultures. With dried blood, as stated above, a solution of the blood is first made according to a known color, and the dilution is made from this. If there is no reaction—that is to say, if within five minutes no marked change is noted in the motility of the bacilli, and no clumping occurs—nothing more is needed; the result is negative. If marked clumping and immobilization of the bacilli immediately begin and become complete within five minutes, this is termed a *marked immediate typhoid reaction*, and no further test is considered necessary, though it is always advisable to confirm the reaction with higher dilutions up to 50 or more, so as to measure the exact strength of the reaction. If in the 1 to 20 dilution a complete reaction takes place within thirty minutes, the blood is considered to have come from a case of typhoid infection, while if a less complete reaction occurs it is considered that only a probability of typhoid infection has been established. By many the time allowed for the development of the reaction with the high dilutions is from one to two hours, but to us twenty minutes with the comparatively low dilution of 1 to 20 seems safer and more convenient. Positive results obtained in this way may be considered conclusive, unless there be grounds for suspecting that the reaction may be due to a previous fairly recent attack or to recent vaccination. The failure of the reaction in one examination by no means excludes the presence of typhoid infection. If the case clinically remains doubtful, the examination should be repeated every few days.

**Use of Dead Cultures.**—(See chapter on Agglutination).

**Proportion of Cases of Typhoid Fever in which a Definite Reaction Occurs, and the Time of its Appearance.**—As the result of a large number of cases examined in the Health Department Laboratories, it was found that about 20 per cent. give positive results in the first week, 60 per cent. in the second week, about 80 per cent. in the third week, about 90 per cent. in the fourth week, and about 75 per cent. in the second month of the disease. In 98 per cent. of the cases in which repeated examinations were made (hospital cases) a definite typhoid reaction was present at some time during the illness.

**Persistence of the Reaction after Convalescence or after Immunization.**—A definite typhoid reaction has been observed from three months to a year after convalescence, and a slight reaction, though much less than sufficient to establish a diagnosis of typhoid infection, from one to fifteen years after the disease. In persons in whom the typhoid bacilli persist the serum reaction may last as long as the bacilli remain in the body. As a rule the reaction becomes progressively weaker during the first six months after vaccination, and has usually completely disappeared at the end of a year.

**Diagnostic Value of Widal Reaction after Vaccination.**—As agglutinins may persist even a year or longer in some individuals one can never be certain as to the interpretation to be placed on a positive reaction in the vaccinated. As already stated Dreyer believes that this difficulty may be overcome by determining whether or not the agglutinins rise or fall, which indicates an active infection. Blood cultures is the better method when cases are seen early.

**Typhoid Bacilli in the Blood.**—A blood culture is usually positive during the first week of typhoid fever, and is the best method for early diagnosis. Bacilli appear in the blood even in the first few days of the disease. In the first week, nearly 100 per cent. positive results are obtained, in the second week 50 per cent. and then progressively less till the end of the disease. In relapses they reappear.

**Method.**—The blood is drawn from the median basilic vein by syringe, and is inoculated into either broth or bile medium. If broth is used, several flasks are used with sufficient broth to dilute the blood fifty times or more. The essential thing is to dilute the blood sufficiently so that coagulation does not occur. The separated serum apparently is more strongly bactericidal and inhibits growth.

Bile mediums are more convenient, as the bulk need not be so large. The bile inhibits coagulation. Three parts of bile medium are used to one part of blood. The bile is plated on Endo, or Conradi, after eighteen hours' incubation. It should also be examined after two or three days, as growth is sometimes delayed. When blood serum is collected for the agglutination reaction, the clot inoculated into bile medium will frequently yield a positive reaction.

Another method, not as satisfactory as the preceding, but valuable in emergencies, is to inoculate the blood into ten times its volume of distilled water. Hemolysis results and the proteins supply the nutrient substances for growth.

**Typhoid Bacilli in Feces.**—The following table by Schröder gives the number and time of positive results that could be expected in routine work:

| | No. examined. | Positive. | Per cent. |
|---|---|---|---|
| First week | 115 | 11 | 9.6 |
| Second week | 160 | 45 | 28.1 |
| Third week | 71 | 16 | 22.5 |
| Fourth week | 41 | 9 | 21.9 |
| Fifth week | 26 | 4 | 15.3 |
| Sixth week | 12 | 2 | 16.6 |
| Convalescent | 190 | 10 | 7.9 |

The more promptly the stools are plated after passage, the better is the chance of obtaining typhoid bacilli. Hiss, examining the stools very quickly after passage, obtained a very high percentage of positive results, in 43 consecutive cases, 37 of which were in the febrile stage and 6 convalescent. In a number of instances only one stool was examined, but even under these adverse conditions the average of positive results in the febrile stage was 66.6 per cent. Out of 26 cases of typhoid fever examined in hospitals, 21 were in the febrile stage and 5 convalescent. In 17 of the febrile cases the presence of typhoid bacilli, often in great numbers, was demonstrated. Thus in these carefully followed cases the statistics show over 80 per cent. of the febrile cases positive. The bacilli were isolated from these cases as early as the sixth day, and as late as the thirtieth day, and in a case of relapse on the forty-seventh day of the disease. These observations, with regard to the appearance of the bacilli in the stools during the febrile stage and their usually quick disappearance, except in the permanent typhoid carriers, after the defervescence, have been confirmed by others. We have, however, a carrier under observation who was discharged from the hospital after three negative examinations. The bacilli are isolated in some cases before the appearance of the Widal reaction; and in some cases in which no Widal reaction was demonstrated. Between the seventh and twenty-first day of the disease, experience seems to indicate that the bacilli may be obtained from about 25 per cent. of all cases on the first examination and from about 75 per cent. after repeated examinations. In some samples of feces typhoid bacilli die out or are overgrown within twenty-four hours; in others they remain alive for days or even weeks. This seems to depend on the bacteria present in the feces and upon its chemical character. Probably the presence of typhoid bacilli in some stools and their absence in others must be explained largely by the characteristics of the intestinal contents. The short life of the typhoid bacillus in many specimens of feces suggests that stools be examined as quickly as possible. In fact, unless the physician wishes to take the trouble to have the sample of feces sent immediately to the laboratory, it is hardly worth while for the bacteriologist to take the trouble to make the test. (When specimens are delayed in transit the method suggested by Teague and Clurman[1] can be employed. One part of feces should be added if fluid, or well rubbed up if solid, in two parts of 30 per cent. glycerin in 0.6 per cent. salt solution. This concentration of glycerin only slowly affects the typhoid bacillus but prevents its overgrowth by the fecal bacteria.)

**Method.**—The feces, if solid, are rubbed up with peptone-water, otherwise they can be used without further preparation. The fluidified feces should consist of a mixture of a generous amount of the whole mass, not merely a loopful.

The density of the suspension to employ is a matter of experience or successive dilutions may be plated. For general use the Endo or Conradi medium are most frequently employed. We employ Endo and a brilliant green agar, using two concentrations of brilliant green (see page 126) which by suppression of some or all of the fecal flora allows a heavy inoculation with coincident increase in positive results. None of the fluid enrichment media that we have tried have been satisfactory for general use, although they may be successful in isolated cases. We have had no experience with the published method of Teague and Clurman which may yield better results. Plates are streaked (see page 133) and the colonies of typhoid can then be agglutinated directly either microscopically or by using the slide method. In the latter a drop of saline for control and a drop of high-titer serum are placed on a slide and the colony rubbed up in the saline and then in the serum. With appropriate dilutions of the serum, agglutination will take place almost immediately.

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[1] Jour. Infect. Dis., 1916, xviii, 653; Jour. Med. Res., 1916, xxxv, 107.

For fishing, Russell's medium is used, which gives a tentative indication of the nature of the organism, which can be confirmed by agglutination and further cultural tests.

**Typhoid Bacilli in the Urine.**—Of great interest is the frequent occurrence of typhoid bacilli in large numbers in the urine. The results of the examinations of others as well as our own indicate that the typhoid bacilli are not apt to be found in the urine until the end of the second week of the fever, and may not appear until much later. From this on to convalescence they appear in about 25 to 50 per cent. of the cases, usually in pure culture and in enormous numbers, even as high as 1,000,000 per cubic centimeter. They are found until several weeks or months after convalescence; in exceptional cases they persist for years. When we think of the chances such cases have to spread infection as they pass from place to place, we begin to realize how epidemics can start without apparent cause. The more we investigate the persistence of bacteria in convalescent cases of disease, the more difficult the prevention of their dissemination is seen to be. The disinfection of the urine should always be looked after in typhoid fever, and convalescents should not be allowed to go to places where contamination of the water supply is possible, without at least warning them of the necessity of great care in disinfecting their urine and feces for some weeks.

**Method.**—If the bacilli are numerous, as evidenced by examination in the hanging drop, they are easily obtained by direct plating, otherwise the urine should be centrifuged and the sediment plated, and larger amounts inoculated into bile medium for enrichment.

**Typhoid Bacilli in Rose Spots and Spleen.**—Although the bacilli have been frequently isolated from rose spots, it is a less convenient method than blood cultures. Spleen puncture has been employed and although cultures are usually positive the operation is dangerous, and has been abandoned.

**Detection of Typhoid Bacilli in Water.**—There is absolutely no doubt that the contamination of streams and reservoirs is a frequent cause of the outbreak of epidemics of typhoid fever, but the actual finding and isolation of the bacilli is a very rare occurrence. This is often due to the fact that the contamination has passed away before the bacteriological examination is undertaken, and also to the great difficulties encountered in detecting a few typhoid bacilli when they are associated with large numbers of other bacteria.

**The Importance of Ice in the Production of Typhoid Fever.**—The total number of instances of typhoid fever which have been reasonably attributed to ice infection are remarkably few. One was in France, where a group of officers placed ice made from water polluted by a sewer in their wine and afterward a large percentage developed typhoid fever, while those of the same company not using ice escaped. A second instance was a small epidemic which occurred among those who used ice from a pond. It was found that water directly infected with typhoid feces had flowed over its frozen surface and been congealed there.

The fact that freezing kills a large percentage of typhoid bacilli makes it indeed possible to conceive that ice from moderately infected water while still polluted contains so few living typhoid bacilli that only the exceptional person here and there becomes infected, and so the source of the infection remains undetected.

If this be true and scattered cases occur, there should be at least some increase in some if not every year in March, April, and May in such a city as New York, where four-fifths of all the ice consumed is from the Hudson River, which is known to be contaminated with typhoid bacilli. Many persons place the ice directly in their drinking water.

When we examine the records for the past ten years we find no increase of typhoid fever in Greater New York during those months, with the one exception of 1907, when we had in the borough of Manhattan a sharp outbreak lasting four weeks. This outbreak did not occur at all in Brooklyn. As the people of Brooklyn drank different water, but received ice from the same places of the Hudson River as those of Manhattan, this directed attention to the water or milk rather than the ice. Examination of the Croton watershed at the time showed that a small epidemic of typhoid existed there and that pollution of the water was probable. This suggested still more strongly that the water and not the ice was the cause of the typhoid infection.

It happened that most of the cases occurred in those living in the section of the upper West Side, where only well-to-do people live. An investigation showed that the majority of the infected had used only artificial ice and several had used no ice in their water at all.

**Life of the Typhoid Bacillus in Ice in Laboratory Experiments.**—The first important investigation was that of Prudden, who showed that typhoid bacilli might live for three months or longer in ice. This experiment is frequently wrongly interpreted, as when a recent writer states: "It has been amply demonstrated that the germs of typhoid fever are not killed by freezing and that they have been known to live in ice for long periods of time."

It is true that in Prudden's experiment a few typhoid bacilli remained alive for three months, when the experiment was terminated, but those were but a small fraction of 1 per cent. of the original number. Following Prudden's experiment Sedgwick and Winslow, in Boston, and Park, in New York City, carried on independently a series of experiments. These led to the same conclusions. A table summarizing a final experiment of ours in which twenty-one different strains, mostly of recent isolation, were subjected to the test is given below:

LIFE OF TWENTY-ONE STRAINS OF TYPHOID BACILLI IN ICE.

| | Average number of bacilli in 1 gm. of ice. | Percentage typhoid bacilli living. |
|---|---|---|
| Before freezing | 2,560,410 | 100.0 |
| Frozen three days | 1,089,470 | 42.0 |
| Frozen seven days | 361,136 | 14.0 |
| Frozen fourteen days | 203,300 | 8.0 |
| Frozen twenty-one days | 10,280 | 0.4 |
| Frozen five weeks | 2,950 | 0.1 |
| Frozen nine weeks | 127 | 0.005 |
| Frozen sixteen weeks | 107 | 0.004 |
| Frozen twenty-two weeks | 0 | 0.0 |

In these experiments twenty-one different flasks of Croton water were inoculated each with a different strain of typhoid bacilli. In one a little of the feces rich in typhoid was directly added. The infected water in each flask was then pipetted into thirty tubes. These tubes were placed in a cold-storage room in which the temperature varied from 20° to 28° F. At first tubes were removed and tested twice a week, later once a week. The reason for using so many different strains was because it has become evident that some cultures live longer than others.

At the end of five weeks the water infected with six cultures was sterile, at the end of sixteen weeks only four strains remained alive.

Interesting investigations of Hudson River ice were carried out in 1907 by North.

There was noticed a considerable difference between the number of bacteria in the top, middle, and bottom layers of ice. This is natural, since while water in freezing from above downward markedly purifies itself, 75 per cent. of the solids and a fair proportion of bacteria being eliminated, yet this cannot happen in the case of the snow blanket which becomes flooded by rain or by cutting holes through the ice. Here all impurities, such as dust and leaves which have fallen on the surface and dirt which may come from the water, remain with the bacteria which they carry, since all are retained in the porous snow. The bacteria in freshly cut bottom ice generally show the least destruction by freezing.

The river water in the forty specimens averaged 1800 bacteria per cubic centimeter, the top ice 306, the bottom ice 36 and the middle ice 14. Only four specimens of top ice had over 500 bacteria per cubic centimeter; none of the specimens of middle or bottom ice.

The great destruction by freezing is noticeable in these figures. Even the top ice soiled by flooding and by the horses and men gathering it contained but 16 per cent. as many bacteria as the water from which it was obtained. The bottom ice, the last to be frozen, had but 2 per cent. of those in the water.

**Conclusions in Regard to Ice Pollution.**—The danger from the use of ice produced from polluted water is always much less than the use of the water itself. Every week that the ice is stored the danger becomes less, so that at four weeks it has become as much purified from typhoid bacilli as if subjected to sand filtration. At the end of four months the danger becomes almost negligible, and at the end of six months quite so.

**Differential Diagnosis.**—The typhoid bacillus is easily separated from the other members of the group, and for practical purposes it is sufficient identification if the colony on the special plating media and the growth on Russell's medium is characteristic and is agglutinated in relatively high dilutions of a serum, so as to eliminate the action of group agglutinins. If a strain does not agglutinate freely, as commonly happens with strains isolated from the blood, it should be transferred daily on plain agar and again tested. The range of group agglutinins in the serum used should be known or error will result.

In important examinations or strains from unusual sources as water, etc., a more extensive cultural study should be done. Likewise, absorption of agglutinins and possibly the production of agglutinins for a known strain should be tested.

**B. (Fecalis) Alkaligenes.**—This group resembles the typhoid bacillus but produces acid from no sugars. It is frequently present in the intestines. It is usually only a saprophyte, but has been found in a few cases of disease in man.

Other non-gasproducing typhoid-like bacilli have been isolated not only from the feces of man but also from feces of cholera-infected swine, cow's feces and water.

# CHAPTER XXIV.

## PARATYPHOID-ENTERITIDIS GROUP.

GÄRTNER, in 1888, found a bacillus in association with a meat-poisoning epidemic. He named it Bacillus enteritidis. A cow sick with diarrhea had been slaughtered and the meat sold for food. Of the persons eating the meat fifty-seven became ill.

Similar bacilli were isolated by Smith from swine suffering from hog cholera and from mice by Löffler. These bacilli became known as the hog cholera group. Similar bacilli were also isolated by others from human cases resembling typhoid fever. Schottmüller reported in 1900 that he was able to differentiate paratyphoid strains into two groups, one differing slightly from B. typhosus and the other considerably. The work of Durham and Buxton gave the basis for a further classification into subgroups.

Numerous types have been isolated from infections in man and animals, and most of the terms in use refer to their source, this not necessarily a factor in the ultimate classification. Gas production from glucose is usually given as the differential characteristic between the paratyphoid bacilli and B. typhosus. This characteristic is susceptible to variation and may be lost. From our own work it would appear that the ability to ferment rhamnose is a basic characteristic of the paratyphoid-enteritidis group. The more the strain resembles the typhoid bacillus, however (especially the non-gasproducing type mentioned above), the lower is the avidity for this carbohydrate. The ability to ferment salicin or saccharose, serves to exclude many of the paratyphoid-like organisms found in feces. Indol also is not produced by the types in this group known to be pathogenic for man.

**Paratyphoid A.—Disease Produced.**—First isolated by Gwyn, later by Schottmüller, Brown and Kayser and others. It produces a typhoid-like disease in man, but is of relatively infrequent occurrence in temperate areas but more frequently encountered in warmer areas. The bacillus may be present in the feces, urine, blood and bile. The postmortem findings have been variable. In a few the typical intestinal lesions of typhoid infections were present, in others they were absent. A diffuse inflammation of the intestines may be found.

**Morphology and Biology.**—Similar to typhoid in many respects. Ford and also Krumwiede[1] have shown that it differs from all other members of the paratyphoid-enteritidis group in that it does not produce acid from xylose. Not all the non-xylosefermenting types from man are alike agglutinatively and the latter observer suggests that all be included in the paratyphoid "A" group and that the strains differing agglutinatively be considered a subgroup. This fermentative reaction is more reliable than the differentiation by litmus milk. The reaction on litmus milk has, since Schottmüller's work, been relied upon for differentiation of B. paratyphosus A from other members of the paratyphoid group. The reaction, however, is only quantitative and exceptions occur.

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[1] Jour. Med. Res., 1916, xxiv, 335.

**Communicability.**—Communicability is the same as for typhoid.

**Diagnosis.**—See below.

**Paratyphoid B.**—This type is now generally acknowledged to be a distinct human pathogen different from the similar paratyphoid types found in animals. If this contention is correct the infection by B. paratyphosus "B" is transferred only from man to man directly or indirectly as in the preceding enteric fevers. As we believe this to be the correct view, the acute infections or intoxications resulting from meat infection or rather intoxication are considered separately.

**Disease Produced in Man.**—Clinically it resembles typhoid fever. As a rule, the bacteriemic character of the disease is more marked.

**Morphology and Biology.**—They closely resemble the other members of this group, but grow more freely and rapidly. For differential characteristics, see pp. 394 and 395.

**Occurrence in Healthy Persons; Paratyphoid Bacillus Carriers ("A" and "B" Types).**—When general invasion occurs the bacilli may be found in the urine after convalescence for shorter or longer periods of time. Its localization in the gall-bladder and its excretion in the feces by chronic carriers is fairly frequent. We recently had the opportunity of examining the feces of every man in a militia regiment which had been badly infected with paratyphoid "A," while at the Mexican border. We found nearly 4 per cent. of healthy carriers. This incidence of normal carriers is interesting in relation to normal carriers of B. typhosus. Is this greater incidence only apparent, due to the greater ease with which we can isolate B. paratyphosis as compared with B. typhosus or actual, because the former known to be less virulent, a greater number of persons escape infection but become carriers for a shorter or longer time? Careful bacteriological examination has revealed paratyphoid-like bacilli in the feces of a considerable percentage of healthy persons. This fact is unfortunately lost sight of, and etiological importance ascribed to such organisms when isolated from the feces during disease. Because of the range of group agglutination, such organisms may even be agglutinated by the patients' serum as well. Such paratyphoid-like types may, however, cause localized infections, especially of the genito-urinary system. Occasionally they cause a terminal septicemia. In a few instances such types have been isolated from the blood stream in infections running the clinical course of typhoid fever. As has been noted above atypical "A" varieties are encountered so that we must conclude that occasionally the enteric type of fever is due to atypical varieties. Such a conclusion, however, in the individual case must be supported by a positive blood culture.

**Frequency of Paratyphoid Infections.**—In this country the disease is relatively infrequent, though statistics are not available. It is frequent in Europe, especially in certain districts. In the present war the disease has been very common. In this country military camps have been favorable places for its development as seen in the outbreak of paratyphoid ("A") fever in the militia at the Mexican border.

**Communicability.**—The individual case and the carrier either through contact or by the contamination of water and milk, etc., are the usual sources of infection for paratyphoid fever.

**Diagnosis of Paratyphoid Fever.**—As with typhoid fever the blood culture is the diagnostic method of choice. A positive blood culture is likely to continue longer than in typhoid fever. The blood culture method is the surest means of making a differential diagnosis, especially if the patient has been vaccinated. The isolation from the urine is also valuable. The presence of bacilli in the feces is not necessarily diagnostic as this may be due to the carrier condition. Differential diagnosis by agglutination gives us only presumptive evidence. The difficulties of drawing a conclusion are greatly enhanced when vaccination has been carried out. As has been previously noted Dreyer believes that these difficulties may be overcome by determining the rise and fall in agglutinins for one of the types, that is, for B. typhosus and B. paratyphosus "A" or "B." Agglutinins develop later and usually less freely in infections due to B. paratyphosus "A." Where isolation methods are employed the isolated type must be identified, not only culturally (see below), but also by agglutination with known type sera. The methods of isolation given for B. typhosus are directly applicable for the paratyphoid types. The latter are more resistant to brilliant green so that their isolation from feces is relatively easy.

**Immunity.**—Immunization has been very successful during the late war, a triple vaccine containing B. typhosus and B. paratyphosus "A" and "B" being employed. (See Vaccines.)

**Food Poisoning or Infections due to Paratyphoid Types.**—Two varieties of bacilli, are encountered in the domestic food-producing animals, *B. choleræ suis* and *B. enteritidis.* Both are encountered in hogs, the former more frequently. In cattle B. enteriditis is the usual type. These bacilli cause infections which may terminate as a septicemia. In hogs this infection is usually secondary to infection by the virus of hog cholera. If the meat from such animals is used for food disease is produced in man. The type of disease produced depends on the relative proportion of preformed toxins and bacilli in the food. If there is an abundance of the former a "choleraic" form of disease results. Where there is less preformed toxin a milder "gastro-enteric" form of the disease develops. As the toxin is heat-resistant disease may result even in the absence of viable bacilli. Apparently the ingestion of the bacillus alone will lead to gastro-enteric symptoms. The period of incubation is variable and depends on the amount of preformed toxin present. The bacilli may invade the blood stream and be found therefore in the urine. In this case a febrile disease of short duration may develop.

Meats or edible organs from cattle, swine, horse, sheep, geese, as well as various products containing these, are most frequently associated with acute food poisoning or infection. Although contamination of meat may occur in other ways the important source in epidemics is meat from infected animals. Such meat may by contact infect meat from healthy animals. Vegetable foods have also caused this type of infection due probably to contamination by infected meats. As B. paratyphosus "B" has the same ability to produce toxic substances it may apparently cause food poisoning. In fact one food-poisoning outbreak has been attributed to the contamination of food by a B. paratyphosus "B" carrier.

*Diagnosis.*—The isolation and identification by cultural and agglutination reaction of the offending type from the food or from the infected individual makes diagnosis certain. A positive agglutination reaction is of some value a week or later after the infection or poisoning. If the food had been sufficiently cooked to kill all the bacilli this may be the only means of making a presumptive diagnosis. Probably a reaction of 1 to 20 to 1 to 40 could be considered diagnostic. Both varieties of bacilli should be employed in the test.

## SUMMARY OF MEMBERS OF THE PARATYPHOID-ENTERITIDIS GROUP.[1]

*B. paratyphosus A and B. paratyphosus B.*—Causative agents in paratyphoid fever in man.

*B. sanguinarium.*—So-called fowl typhoid bacillus.

*B. pullorum.*—Causative agent of white diarrhea in chicks.

*B. abortus equi.*—Causative agent in infectious abortion in mares.

*B. choleræ suis.*—A group encountered in secondary infections in hogs. Besides the type organism there are probably many subvarieties. B. suipestifer, swine-typhus bacillus are other terms.

*B. typhi murium.*—A name based on the source, viz., infections in mice. Probably many varieties cause septicemic infections in these animals.

*B. psittacosis.*—Cause of enteritis in parrots, infectious for man, resulting disease called psitticosis. Data is limited. Position in group not determined.

*B. pestis caviæ*—A distinct type of bacillus causing "pseudotuberculosis" lesion in guinea-pigs. This type of infection is important as the lesions may be confused with those of the tubercle bacillus. Recently this lesion developed in a series of our guinea-pigs inoculated with milk to determine the presence of tubercle bacilli, thus vitiating the results. Some of the so-called typhi murium types belong to this group of bacilli.

*B. enteritidis.*—Encountered in domestic food-producing animals. Common in rodents: The strains from the latter source frequently called B. danysz. Rats are frequently carriers. This bacillus as well as the typhi murium varieties have been used as viruses for the destruction of rodents. They are marketed under the name of Ratin, Liverpool Virus, Danysz Virus, etc. Foods should be carefully protected from contamination by such viruses.

*B. paratyphosus "C."*—A term employed for the miscellaneous types which do not fall into the definite groups. Its use should be condemned, especially when applied to the paratyphoid-like types in feces, implying etiological importance to the presence of such types without presenting any confirmatory data.

Besides the above definite members of this pathogenic group, there exist many paratyphoid-like types, some of which are occasionally encountered, as already noted, in local or general infections.

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[1] See Krumwiede, Kohn and Valentine: Jour. Med. Res., 1918, xxxviii, 89; 1919, xxxix, 449, for details and references; also Ten Broeck: Jour. Exp. Med., 1918, xxviii, 759.

There are also encountered bacilli which for want of better classification or terminology are called "intermediates." They resemble the paratyphoid bacillus culturally but one or more aberrant characteristics show their relationship to B. coli. Thus they may ferment saccharose, lactose (after a delay), salicin, etc. The term paratyphoid bacillus is a misnomer when applied to this group.

*Cultural Reactions.*—Considerable confusion and differences in opinion are encountered when one studies the results of various observers. To a large extent the results of *later* studies indicate that this was not only due to a recognized tendency to variation but also to an avidity factor, overlooked by most workers. This avidity factor, that is, the differences in fermentative capacity exhibited by members of the same group or even by otherwise identical types is brought out in the following table:

MAIN DIFFERENTIAL CULTURAL CHARACTERISTICS.

| Groups or types. | Glucose serum water. | Lead acetate agar. | Gas from glucose. | Acid Production from.[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Rhamnose. | Maltose. | Xylose. | Arabinose. | Dulcit. | Inosite. |
| B. typhosus . . . | + | +(± or −) | − | − | + | + or ± | − or ± | ± or − | − |
| B. sanguinarium . | irreg. | irreg. | − | ± | + | + or ± | + | + or ± | − |
| B. pullorum . . | " | " | +(−)[1] | + | − | + or ± | + | − | − |
| B. paratyphosus "A" | + | − | + | + | + | − | + | ± | − |
| B. abortus equi. . | + | − | + | + | + | + | + | + | − |
| B. chol. suis . . | + | − | +(−)[1] | + | + | + | − to ± | − to + | − |
| B. chol. suis (?)[2] . | − | + | + | + | + | + | + | + | + |
| B. paratyphosis "B" | − | + | +(−)[1] | + | + | + | + | + | + or −[3] |
| B. typhi murium (?) | − | + | + | + | + | + | + | + | + |
| B. pestis caviæ . . | − | + | + | + | + | + | + | + | + or −[3] |
| B. enteritidis . . | − | + | + | + | + | + | + | + | − |

Symbols: Glucose serum water, + = pink, no reduction.
− = pale or nearly colorless reduction.
Lead acetate agar, + = browning, sulphide production.
− = no change.
Fermentation, + = prompt, ± delayed, − negative.

Notes:
1. Exceptional findings.
2. Agglutinatively different from B. paratyphosis B. Some of these strains may be variants, having acquired these characteristics. A few recently isolated strains have been found by Ten Broeck to give these reactions (inosite not tested). He tentatively suggests the name swine-typhus.
3. Strains showing these differences may be identical agglutinatively and on agglutinin absorption. All freshly isolated "B" types have fermented this substance.
4. All produce acid from glucose, galactose, levulose and mannite. None produce acid from lactose, saccharose, raffinose, erythrite, adonite, salicin or inulin.

Media: Carbohydrates, 1 per cent. in peptone water, Andrade indicator.

Glucose Serum Water: Horse serum, 1 part; distilled water, 4 parts, glucose, 0.1 per cent.; Andrade indicator, 1 per cent. Steam twenty minutes for two successive days.

Lead Acetate Agar: Cool agar (in bath) to 50° to 60° C., add sufficient 0.25 per cent. aqueous solution of basic lead acetate to give 0.05 per cent. concentration; inoculate by stab between agar and glass. Test suitability with known strains.

# CHAPTER XXV.

## DYSENTERY GROUP.

Dysentery may be divided into acute and chronic. Amebæ appear to be the chief exciting factor in most cases of chronic dysentery, though bacilli of the colon group also play a part.

In temperate climates acute dysentery is but very rarely due to amebæ, but usually to the bacilli identified by Shiga or to allied strains later identified by Kruse, Flexner, Park, Hiss, Strong and others. The usual summer diarrheas are not excited by the dysentery bacilli.

**Historical Note.**—In 1897 Shiga found in the stools of cases of dysentery a bacillus which had not been before identified. In 1900 Flexner and Strong isolated bacilli which they at that time considered the same as those isolated by Shiga. In the same year Kruse, in Germany, isolated bacilli from cases of asylum dysentery which differed, however, in their agglutinative properties. Park and Dunham (1902) isolated a bacillus from a severe case of dysentery during an epidemic at Seal Harbor, Mt. Desert, Maine, which they showed differed from the Shiga dysentery bacillus in that it produced indol and differed in agglutinating characteristics. At first they considered it the same as the Flexner strain, but it was shown later by Park to be a distinct variety, and later found by him in widely separated epidemics.

Martini and Lentz, in December, 1902, found that the Shiga type was present in separate epidemics in Europe, but also in some cases, that other types similar to those isolated by Flexner, Park, Kruse, and others were found. These types differed from the Shiga type in fermenting mannite and in agglutination. In January, 1903, Hiss and Russel showed that a strain isolated by them differed from the Shiga type in the same characteristics as the Park-Dunham culture.

German observers at first were inclined to consider the Shiga type as the only one producing dysentery, while the American observers considered both types of equal importance. Park investigated several epidemics and isolated only the Shiga type from some, from others either the Park-Hiss or the Flexner types, thus definitely proving the importance of the mannite fermenting types. The results obtained by others later were the same, so that no doubt exists that all of the types produce true dysentery.

**Morphological and Cultural Characteristics of Dysentery Bacilli.**—**Microscopic.**—Similar to bacilli of the colon group.

**Staining.**—Similar to bacilli of the colon group.

**Motility.**—No definite motility has been observed. The molecular movement is very active. Flagella are absent.

**Appearance of Cultures.**—On *gelatin* the colonies appear more like the typhoid than the colon bacilli. Gelatin is not liquefied. On agar growth is somewhat more delicate than that of the average colon cultures.

*On Potato.*—A delicate growth just visible or distinctly brownish.

*In Bouillon.*—Diffuse cloudiness with slight deposit and sometimes a pellicle.

See page 367 for comparison with other members of colon-typhoid group.

The fermentation reactions vary, and on this basis the dysentery group is divided into subgroups and types. The first three groups are encountered in the United States. The last type, if it exists, is of infrequent occurrence. It is accepted as a separate type by Shiga and by Lentz.

| | Glucose. | Mannite. | Maltose. | Saccharose. | Indol. |
|---|---|---|---|---|---|
| B. dysenteriæ | + | − | − | − | − |
| B. paradysenteriæ: | | | | | |
| Type 1 (Park-Hiss) | + | + | − | − | + |
| Type 2 (Flexner) | + | + | + | − | + |
| Type 3 (Strong) | + | + | − | + | + |

In differential tests 2 per cent. mannite and 2.5 per cent. maltose give surer and more prompt reactions than the usual 1 per cent. The fermentations as given are only of differential value with freshly isolated cultures. After artificial cultivation as noted by Hiss and by Lentz the Park-Hiss strains may ferment maltose, and the Flexner strains, saccharose. According to Lentz animal passage will usually restore the original characteristics.

The carbohydrate fermentations as given are based on twenty-four observations and are accepted as a reliable means of differentiation by many observers. Their reliability has been questioned by others, some claiming that many groups exist if the fermentative reactions be considered as the criterion of differentiation. In our original work the fermentative differentiation coincided with that obtained by agglutination. The strains isolated, however, came from epidemics and it is possible that had we studied more strains from sporadic cases this parallelism might not have been so complete. We have found that when strains have been in cultivation for some time many apparent groups can be separated especially if incubation and observations be continued up to the twelfth day. The apparent grouping will change as the delayed fermentation becomes evident. Certain fermentative differences, with arabinose and sorbite[1] for instance, are apparently uncorrelated, being positive or negative among strains of the same group.

The more recent work of Sonne seems to indicate very strongly that even with relatively freshly isolated strains, the fermentative differences are not reliable for classification. He also found a group of paradysentery bacilli different from those given above. His suggested classification of the paradysentery bacilli is as follows.

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[1] Also observed by English workers with sorbite.

GROUP I.—*Park-Hiss types* (this group will include some cultures giving the fermentative reaction usually attributed to the Flexner type).

GROUP II.—*Flexner* (includes so-called Strong type; some cultures may have fermentative characteristics usually attributed to the Park-Hiss types).

GROUP III.—*Sonne* (includes types with so-called Flexner characteristics).

It is evident from the limited facts given that he places no reliance on fermentative differentiation. For instance, he found the following apparent groupings according to the time of incubation:

24 hours—Park-Hiss. 42; Flexner, 32 } Total,
48 hours—Park-Hiss, 31; Flexner, 43 } 74 strains.

He also gives tables showing changes in fermentative characteristics on successive tests at considerable periods.

His actual differentiation is based on agglutination. He isolated a few strains which apparently did not fall into the above groups. This failure may have been due to relative inagglutinability or they may be aberrant strains or even not dysentery bacilli at all.

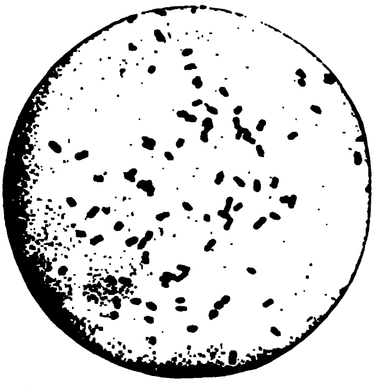

FIG. 139.—Dysentery bacilli. × 1000 diameters.

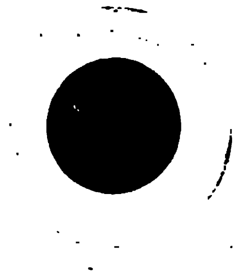

FIG. 140.—Colony of dysentery bacilli in gelatin. × 40 diameters.

We are inclined to believe that in this group as in the paratyphoid group, some of the fermentative differences are not basically qualitative but rather quantitative. That is, a fermentative capacity may be potential or latent in a group and appear irregularly, depending on time of cultivation, character of medium and time of observations. It is possible that in epidemics as in passage experiments this latency is more marked, resulting in sharper differential results. In the more sporadic infections, it is reasonable to believe that this adaptation will be less marked and greater irregularities therefore encountered. At least there is no justification for differentiation of stock cultures on the basis of cultural reactions, a fact apparently little appreciated.

**Pathogenesis.—Animal Tests.**—No characteristic lesions with one exception have followed the feeding of large quantities of bacilli. Dogs at times have diarrhea with slimy stools, but autopsy shows merely a hyperemia of the small intestine. The disease can be produced and occurs spontaneously in monkeys. Many animals are very sensitive to dead or living bacilli injected into the subcutaneous tissues, vein or peritoneal cavity.

The autopsy of animals dying quickly from injection into the peritoneum of living or dead bacilli shows the peritoneum to be hyperemic, the cavity more or less filled with serous or bloody serous exudate. The spleen is sometimes moderately swollen. The small intestine is filled with fluid, the large intestine is usually empty. The mucous membrane of both is hyperemic and sometimes contains hemorrhages. Conradi found ulcer formation in one case.

Subcutaneous injections of dead or living cultures are followed by infiltration of tissues and frequently by abscess formation. The dysentery bacilli are not found in the blood or organs of animals.

**Toxins.**—A highly poisonous endotoxin is found in autolysates and in filtered broth cultures. When injected into animals death results and on autopsy the same lesions are found as following the injection of live or dead bacilli. This endotoxin can be neutralized by large doses of immune serum. Kraus and others claim to have demonstrated the presence of an extracellular toxin against which an antitoxin can be produced. The Shiga type is the more toxic, the others less so or irregularly so.

**In Man.**—The etiological significance of the dysentery bacillus in man is not only shown by its constant presence and by the presence of immune bodies in the serum of infected persons, but also by experimental infection of man. Thus Strong infected two condemned criminals with pure cultures. Jehle infected himself. Kruse reported two accidental laboratory infections, and others have occurred.

As a general rule infection with the Shiga type is more severe and the mortality higher than infection from other types. But the severity of individual cases varies widely during an epidemic.

**Prevalence of the Disease.**—The disease is distributed over the whole world. The Shiga type and Type I of the paradysentery varieties are most commonly found in the United States.

**Character of Disease in Man.**—In the onset, acute dysentery is sudden and ushered in by cramps, diarrhea and tenesmus. Bacillary dysentery is a disease especially of the mucous membrane of the large intestines. The epithelium is chiefly involved. In the lightest cases a catarrhal inflammation alone is present, in the more severe the lymph follicles are swollen and some necrosis of epithelium takes place.

In severe cases in adults the lesions are of a diphtheritic character and may be very marked. The entire lumen of the intestines may be filled with a fibrinous mass of pseudomembrane. In young children, even in fatal cases, the lesions may be more superficial.

**Distribution of the Bacilli.**—The bacilli are only found in the intestines. They do not invade the rest of the body. The feces, therefore, are the only excretions containing them.

**Duration of Life Outside of the Body.**—They usually die in stools in one to two days. In water they die out in several days to a week, exceptionally after a longer time.

**Communicability.**—The infected person is mainly responsible for the spread of dysentery. Especially dangerous are the mild or subacute cases or carriers. Such infection may be direct or it may be indirect through contamination of food, dishes, linen and clothes; water may also be infected and be the cause of epidemics. The bacillus has been isolated from water in such epidemics.

**Bacillus Carriers.**—Both healthy and convalescent carriers are found. The former may be equal to one-fourth or even one-half of the number of cases. Convalescents commonly excrete the bacilli for weeks and some become chronic carriers. The excretion is irregular, and slight relapses occur, when the bacilli are more numerous. The importance of finding and isolating the carriers during an epidemic is obvious. This is not always possible and general precautions against infection should be taken.

**Susceptibility.**—The frequent occurrence of healthy carriers and mild cases along with severe and fatal cases shows the varying resistance to infection. Disturbances of digestion and other conditions lowering the general resistance, such as heat and fatigue, are factors in infection.

**Immunity.**—Immune bodies appear in the blood some time after infection. In animals, immune bodies are also produced by injection. Agglutinins, bactericidal substances, precipitins and opsonins are found. Neutralization of the toxic products is also possible with immune sera. True antitoxin is also probably produced.

Active immunity in animals is produced with difficulty against the Shiga type because of its high toxicity. Against the other types, however, immunity is more easily produced.

**Vaccines and Serums.**—See Part III.

**Diagnosis.**—The use of the Widal reaction is limited, because the symptoms are sufficiently diagnostic in well-marked cases and, further, the reaction does not appear during the early acute stage. Later it may be used. Group or normal agglutinins are commonly present and interfere greatly with the value of the reaction in the paradysentery types. The action on the infecting subgroup is usually sharp, but infection by the types of the paradysentery group can frequently not be differentiated. An agglutination reaction is commonly present in chronic carriers. For even moderate diagnostic value the serum should agglutinate in 1 to 50 in infections with *B. dysenteriæ* and in 1 to 100 in other types.

Isolation of the bacillus is the only method of diagnosis for certain identification of the type causing the infection. The mucous flakes in stools should be selected for plating. The methods of isolation and identification are the same as for typhoid or paratyphoid, except that the crystal violet should be omitted from the Conradi medium as the growth of many strains is inhibited more or less by anilin dyes. In acute cases the bacilli are so abundant in the mucus of most cases that they can be readily isolated from nutrient agar plates. The isolation from carriers is not always easy. If Endo is used the plates should be fresh and the reaction not too alkaline. When the color returns the growth of the Shiga types is usually inhibited as shown by Krumwiede. Veal infusion on a peptic digest basis seems to be more desirable for this group.

*Differential Diagnosis of Type.*—The cultural differences have been given. The actual nature of the bacillus should be verified by agglutination, although the cultural characters may be used for a tentative diagnosis from a case clinically typical.

**Agglutination.**—Sera from rabbits or goats are used, as horses develop normal and group agglutinins to a high degree.

For practical purposes two sera, one for B. dysenteriæ and a polyvalent serum for the paradysentery types may be used. For differentiation of the subgroup monovalent sera are necessary. Park-Hiss and Flexner sera will contain considerable group agglutinins for each other. It is obvious in the light of the discussion on fermentative reactions that especial care be taken to determine the actual identity of strains to be utilized for serum production. It is desirable that serum for the Sonne type be included to determine whether his classification is valid for the strains obtained in this country. Each serum must be carefully titrated (see Agglutinins) or many errors will result. As dysentery strains vary widely in their agglutinability failure of identification due to relative inagglutinability should be followed by repetitions of the test. If necessary absorption may be resorted to.

As we have shown certain anaërogenic colon-like organisms are occasionally encountered which are agglutinated in high dilutions of dysentery sera. These may be excluded if they possess microscopic or cultural characteristics known not to be possessed by the dysentery bacillus. If necessary agglutinin absorption may be resorted to.

# CHAPTER XXVI.

# BACILLUS PYOCYANEUS (BACILLUS OF GREEN AND OF BLUE PUS). BACILLUS PROTEUS (VULGARIS).

## BACILLUS PYOCYANEUS.

The blue and green coloration which is occasionally found to accompany the purulent discharges from open wounds is usually due to the action of the *Bacillus pyocyaneus*. It was first obtained in pure culture and its significance noted by Gessard, 1882.

**Morphology.**—Slender rods from 0.3μ to 1μ broad and from 2μ to 6μ long; frequently united in pairs or in chains of four to six elements; occasionally growing out into long filaments and twisted spirals. The bacillus is actively motile, a single flagellum being attached to one end. Does not form spores. *Stains* with the ordinary anilin colors; does not stain with Gram's stain. Stained with alkaline methylene blue it may look a little like a small diphtheria bacillus.

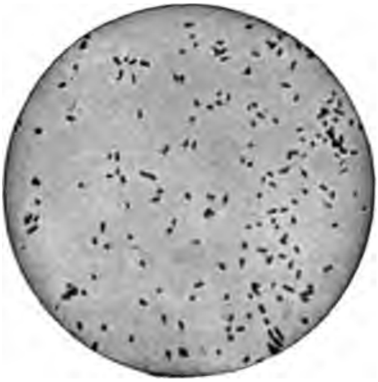

Fig. 141. — Bacillus pyocyaneus. (From Kolle and Wassermann.)

**Biology.** — Aërobic, facultative anaërobic, liquefying, motile bacillus. Growing anaërobically it produces no pigment. Grows readily on all artificial culture media at room temperature, though best at 37° C., and gives to some of them a bright green color in the presence of oxygen. In *gelatin-plate* cultures the colonies are rapidly developed, imparting to the medium a fluorescent green color; liquefaction begins at the end of two or three days, and by the fifth day the gelatin is usually entirely liquefied. The deep colonies, before liquefaction sets in, appear as round, granular masses with scalloped margins, having a yellowish-green color; the surface colonies have a darker green center, surrounded by a delicate, radiating zone. In *stick cultures in gelatin*, liquefaction occurs at first near the surface, in the form of a small funnel and gradually extends downward; later the liquefied gelatin is separated from the solid part of the medium by a horizontal plane, a greenish-yellow color being imparted to that portion which is in contact with the air. On *agar* a wrinkled, moist, greenish-white layer is developed, while the surrounding medium is bright green; this subsequently becomes darker in color, changing to blue green or almost black. In *bouillon* the green color is produced, and the growth appears as a delicate, flocculent sediment. *Milk* is coagulated and assumes a yellowish-green color.

**Pigment.**—Two pigments are produced by this bacillus—one of a fluorescent green which is common to many bacteria. This is soluble in water but not in chloroform. The other (pyocyanin) of a blue color is soluble in chloroform, and may be obtained from pure solution in long, blue needles. This pigment distinguishes the *Bacillus pyocyaneus* from other fluorescing bacteria. Pigment production is usually more marked with incubation at 22° C. The ability to produce pigment may be lessened or lost by artificial cultivation.

**Ferment.**—Besides the ferment causing liquefaction of gelatin there is one which acts on albumin. It resists heat. This ferment called pyocyanase is able to dissolve bacteria, and it has been stated to have some protective power when injected into animals. It has been used locally in diphtheria in a number of cases. We think it has no advantage over cleansing preparations.

**Distribution.**—This bacillus is very widely distributed in nature, it is frequently found on the healthy skin of man, in the feces of many animals, in water contaminated by animal or human material, in purulent discharges, and in serous wound secretions.

**Pathogenesis.**—Its pathogenic effects on animals have been carefully studied, especially on guinea-pigs and rabbits. Subcutaneous or intraperitoneal injections of 1 c.c. or more of a bouillon culture usually cause the death of the animal in from twenty-four to thirty-six hours. Subcutaneous inoculations produce an extensive inflammatory edema and purulent infiltration of the tissues; a serofibrinous or purulent peritonitis is induced by the introduction of the bacillus into the peritoneal cavity. The bacilli multiply in the body and may be found in the serous or purulent fluid in the subcutaneous tissues or abdominal cavity, as well as in the blood and various organs. When smaller quantities are injected subcutaneously the animal usually recovers, only a local inflammatory reaction being set up (abscess), and the animal is subsequently immune against a second inoculation with doses which would prove fatal to an unprotected animal. Loew and Emmerich have shown that the enzymes produced in the pyocyaneus cultures are capable of destroying many forms of bacteria in the test-tube, and have a slight protecting value in the body.

Its presence in wounds in man greatly delays the process of repair, and may give rise to a general depression of the vital powers from the absorption of its toxic products. This bacillus has been obtained in pure culture from pus derived from the tympanic cavity in disease of the middle ear, from cases of ophthalmia, and bronchopneumonia. Kruse and Pasquale have found the organism in three cases of idiopathic abscess of the liver, in two of them in immense numbers and in pure culture. Ernst and Schürmayer report the presence of the bacillus pyocyaneous in serous inflammations of the pericardial sac and of the knee-joint. Ehlers gives the history of a disease in two sisters who were attacked simultaneously with fever, albuminuria, and paralysis. It was thought that they would prove to have typhoid fever or meningitis, but on the twelfth day there was an eruption of blisters, from the contents of which the Bacillus pyocyaneus was isolated. Krambals refers to seven cases in which a general pyocyaneus infection occurred, and adds an eighth from his own experience. In this the Bacillus pyocyaneus was obtained postmortem from green pus in the pleural cavity, from serum in the pericardial sac, and from the spleen in pure culture. Schimmelbusch states that a physician injected 0.5 c.c. of sterilized (by heat) culture into his forearm. As a result of this injection, after a few hours he had a slight chill, followed by fever, which at the end of twelve hours reached 38.8° C.; an erysipelatous-like swelling of the forearm occurred, and the glands in the axilla were swollen and painful. Wassermann reports an epidemic of septic infection of the newborn, starting in the umbilicus. In all there were eleven deaths. Lartigau found it in well water, and in great abundance in the intestinal discharges of a number of cases made ill by drinking the water. It has also been found in a certain number of cases of gastro-enteritis in which no special cause of infection could be noted.

We may therefore conclude from these facts that the Bacillus pyocyaneus, although ordinarily but slightly pathogenic for man, may under certain conditions, as in general debility, become a dangerous source of infection. Children would seem to be particularly susceptible.

**Differential Diagnosis of the Pyocyaneus from other Fluorescing Bacteria.**—This is easy enough as long as it retains its pigment-producing property. When an agar culture is agitated with chloroform a blue coloration demonstrates the presence of this bacillus. When the pyocyanin is no longer formed, however, the diagnosis is by no means easy, particularly when the pathogenic properties are also gone.

**Immunity.**—Animal infection is followed by the production of antitoxic and bactericidal substances.

## PROTEUS VULGARIS.

The term Proteus vulgaris is used for a group of bacilli, types of which were first isolated by Hauser in 1885 from putrifying meat. The limits of this group are not well defined. Wenner and Rettger[1] have just reported a systematic study of the proteus group in which they show that several strains classed as different varieties are probably identical and that other strains included in this group do not belong in it. They subdivide the group on the basis of their action on maltose into two species, namely, proteus vulgaris, fermenting the sugar, and proteus mirabilis failing to attack it. The genus cannot be subdivided satisfactorily on the basis of proteolytic action, indol production or agglutinating properties. The following is a description of proteus vulgaris.

**Morphology.**—Bacilli varying greatly in size; most commonly occurring 0.6$\mu$ broad and 1.2$\mu$ long, but shorter and longer forms may also be seen, even growing out into flexible filaments which are sometimes more or less wavy or twisted like braids of hair.

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[1] Jour. Bact., 1919, iv, 331.

The bacillus does not form spores, and *stains* readily with fuchsin or gentian violet. It is Gram-negative.

**Biology.**—An aërobic, facultative anaërobic, liquefying, motile bacillus, which ferments glucose, sucrose and maltose with the production of acid and gas. Grows rapidly in the usual culture media at room temperature.

**Growth on Gelatin.**—The growth upon *gelatin plates* containing 5 per cent. of gelatin is very characteristic. At the end of ten to twelve hours at room temperature small round depressions in the gelatin are observed which contain liquefied gelatin and a whitish mass consisting of bacilli in the center. Under a low-power lens these depressions are seen to be surrounded by a radiating zone composed of two or more layers, outside of which is a zone of a single layer, from which ameba-like processes extend upon the surface of the gelatin. These processes are constantly undergoing changes in their form and position. The young colonies deep down in the gelatin are somewhat more compact, and rounded or hump-backed; later they are covered with soft down; then they form irregular, radiating masses, and simulate the superficial colonies. When the consistency of the medium is more solid, as in 10 per cent. gelatin the liquefaction and migration of surface colonies are more or less retarded. In *gelatin-stick* cultures liquefaction takes place rapidly along the line of puncture, and soon the entire contents of the tube are liquefied.

Upon *nutrient agar* a rapidly spreading, moist, thin, grayish-white layer appears, and migration of the colonies also occurs. *Milk* is coagulated, with the production of acid and the later digestion of the casein.

Cultures in media containing albumin or gelatin have a disagreeable, putrefactive odor, and become alkaline in reaction. Growth is most luxuriant at a temperature of 24° C., but is plentiful also at 37° C. It is an aërobic bacillus but it grows also in the absence of oxygen. In the latter condition it loses its power of liquefying gelatin. It produces indol and phenol from peptone solutions. The proteus develops fairly well in urine, and decomposes urea into carbonate of ammonia.

**Pathogenesis.**—This bacillus is pathogenic for rabbits and guinea-pigs when injected in large quantities into the circulation, the abdominal cavity, or subcutaneously, producing death with symptoms of poisoning. Hauser has obtained the Bacillus proteus (vulgaris) from a case of purulent peritonitis, from purulent puerperal endometritis, and from a phlegmonous inflammation of the hand.

It is probable that in some instances food poisoning has been due to the contamination of foods by B. proteus. Because of the proteolytic power, toxic products "ptomaines" may develop as a result of its growth. Under these conditions decomposition has started and the food is disagreeable both in taste and odor and for this reason food poisoning of this type is probably much more uncommon than that due to members of the paratyphoid-enteritidis group, or to B. botulinus where there is no change or only a slight change in odor and taste.

Proteus vulgaris has been found to be the predominating organism in the alvine discharge in cases of cholera infantum. The prominent symptoms in these cases were drowsiness, stupor, and great reduction in flesh, more or less collapse, frequent vomiting and purging, with watery and generally offensive stools.

The Proteus vulgaris appears to be next in importance to the Bacillus communis in the etiology of cystitis and pyelonephritis.

The Proteus vulgaris is usually a harmless parasite when located in the mucous membrane of the nasal cavities. Here it only decomposes the secretions, with the production of a putrefactive odor. It is found occasionally in the discharge from cases of otitis media in association with other bacteria.

For the use of certain proteus strains in the diagnosis of typhus fever see page 538.

# CHAPTER XXVII.

# THE BACILLUS AND THE BACTERIOLOGY OF TUBERCULOSIS.

**Historical Note.**—A knowledge of phthisis was certainly present among men at the time from which our earliest medical descriptions come. For over two thousand years many of the clearest thinking physicians have considered it a communicable disease; but it is only within comparatively recent times that the infectiousness of tuberculosis has become an established fact in scientific medicine. Villemin, in 1865, by infecting a series of animals through inoculations with tuberculous tissue, showed that tuberculosis might be induced, and that such tissue carried the exciting agent of the disease. He also noticed the difference in virulence between tuberculous material of human and bovine sources, and said that not one of the rabbits inoculated with human material showed such a rapidly progressive and widespread generalization as those receiving material from the cow. Baumgarten demonstrated, early in 1882, bacilli in tissue sections which are now known to have been tubercle bacilli. But these investigations and those of others at the same time, though paving the way to a better knowledge of the disease, proved to be unsatisfactory and incomplete. The announcement of the discovery of the tubercle bacillus was made by Koch in March, 1882. Along with the announcement satisfactory experimental evidence was presented as to its etiological relation to tuberculosis in man and in susceptible animals, and its principal biological characters were given. He submitted his full report in 1884. Innumerable investigators now followed Koch into this field, but their observations served only to confirm his discovery.

**Distribution of Bacilli.**—They are found in the sputum of persons and animals suffering from pulmonary or laryngeal tuberculosis, either free or in the interior of pus cells; in miliary tubercles and fresh caseous masses in the lungs and elsewhere; in recent tuberculous cavities in the lungs; in tuberculous glands, joints, bones, serous effusions, mucous membranes, and skin affections. They are also found in the feces of those suffering from tuberculosis of the intestines or of those who have swallowed tuberculous sputum. They are frequently present in the blood in very small numbers, in large number only in acute miliary tuberculosis.

**Morphology.**—The tubercle bacilli are slender, non-motile rods of about $0.3\mu$ in diameter by $1.5\mu$ to $4\mu$ in length. (Plate VI, Figs. 1 and 2.) The morphology is extremely variable, especially on culture media, and varies with the type of medium used. Commonly they occur singly or in pairs, and are then usually slightly curved; frequently they are observed in smaller or larger bunches. Under exceptional conditions branching and club-shaped forms are observed. The tubercle bacillus is probably closely allied to nocardia. In stained preparations there are often seen unstained portions. In old cultures irregular forms may develop, the rods being occasionally swollen at one end or presenting lateral projections. Here also spherical granules appear which stain with more difficulty than the rest of the bacillus and also retain the stain with greater tenacity. The bacilli, however, containing these bodies are not appreciably more resistant than those not having them; therefore they cannot be considered true spores. The bacilli have a thin capsule, shown in one way by the fact that they appear thicker when stained with fuchsin than with methylene blue.

**Chemical Constituents of the Tubercle Bacilli.**—Water 86 per cent., dry substance 14 per cent., 25 per cent. of which is soluble in alcohol and ether, consisting of free fatty acids and fatty acids combined with the higher alcohol "mykol" to form a wax; lecithin proteins, other nucleo-albumins, and inorganic bases constitute the remainder.

**Staining Peculiarities.**—These are very important, for by them its recognition in microscopic preparations of sputum, etc., is rendered possible. Owing to content of waxy substance it does not readily take up the ordinary anilin colors, but when once stained it is very difficult to decolorize, even by the use of strong acids. The more recently formed bacilli are much more easily stained and decolorized than the older forms. For methods of staining see p. 77.

**Biology.**—The bacillus of tuberculosis is a *parasitic, aërobic, non-motile* bacillus, and grows best at a temperature of about 37° C., limits 30° to 42° C. It does not form true spores.

**Resistance.**—The bacilli, because of the protection given by their waxy substances, it has been assumed, have a somewhat greater resisting power than most other pathogenic bacteria. Frequently a few out of a great number of bacilli resist desiccation at ordinary temperatures for months; most bacilli die, however, soon after drying. There is a greater resistance shown by the tubercle bacillus than by most other non-sporebearing bacilli to the action of the products of bacterial growth as in souring milk, in water, and in sewage. In water and sewage they may remain viable for weeks. They frequently retain their vitality for several weeks, or even months, in putrefying material, such as sputum. In cultures the bacilli do not live longer than three months.

## EXPLANATION OF PLATE VI.

Fig. 1.—Tuberculous lymph node "giant cell" containing tubercle bacilli "human type." Bacilli red, rest of specimen blue. Ziehl-Neelsen stain. × 1000 diam.

Fig. 2.—Tuberculous sputum from human case. Stain same as above. × 1000 diam.

Fig. 3.—Tuberculous sputum, human case. Stained by Hermann's method. Tubercle bacilli violet, rest of specimen brown.

Fig. 4.—Pus from tuberculous abscess in cow. "Bovine type" of bacillus. Stained same as Figs. 1 and 2. × 1000 diam.

Fig. 5.—Section through leprous skin showing bacilli in clumps in and out of cells and large "leprous cell" containing a ball of bacilli. Stained with Ziehl-Neelsen.

Fig. 6.—Photograph of human type of tubercle bacilli from sputum. Bacilli in red, rest of specimen blue. × 1000 diam. (Fränkel and Pfeiffer.)

unless the media be favorable, such as egg or serum; transplants after this time may fail to grow. A few bacilli, sufficient to infect guinea-pigs, may persist much longer. Cold has little effect upon them. When dry, some of the organisms stand dry heat at 100° C. for twenty minutes but are dead in forty-five minutes; but when in fluids and separated as in milk, they are quickly killed—viz., at 60° C. in twenty minutes, at 65° C. in fifteen minutes, at 70° C. the great majority in one minute, all in five minutes, at 80° C. the great majority in one-half minute, all in one minute, and at 95° C. in one-half minute. In some experiments they appear to withstand a higher temperature. As pointed out by Theobald Smith, when milk is heated in a test-tube in the usual way the cream which rises on heating is exposed on its surface to a lower temperature than the rest of the milk, and as this contains a large percentage of the bacteria some of them are exposed to less heat than those in the rest of the fluid. Rosenau points out another source of error: If a moderate number of killed bacilli are injected, limited lesions will arise and caseation may follow. On killing and autopsying the animals, tubercle bacilli can then be demonstrated in smears from the lesions, and the inoculation is considered positive. If, however, this material is reinjected into a second pig, the latter will show nothing on autopsy. This capacity of dead bacilli to cause macroscopic lesions has long been shown by Prudden and Hodenpyl. Its importance, however, is not sufficiently considered.

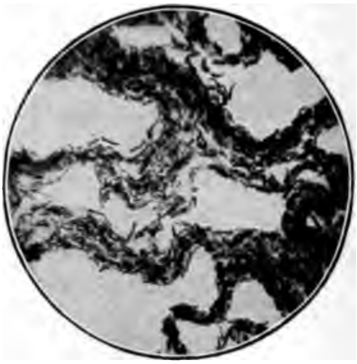

FIG. 142.—Tubercle bacilli. Impression preparation from small colony on coagulated blood serum. × 1000 diameters.

The resisting power of this bacillus to chemical disinfectants, drying, and light is considerable, but not as great as it is apt to appear, for, as in sputum, the bacillus is usually protected by mucus or cell protoplasm from penetration by the germicidal agent. It is not always destroyed by the gastric juice in the stomach, as is shown by successful infection experiments in susceptible animals by feeding them with tubercle bacilli. They are destroyed in sputum in six hours or less by the addition of an equal quantity of a 5 per cent. solution of carbolic acid. Bichloride of mercury is less suitable for the disinfection of sputum as it combines with the mucus and forms a more or less protecting envelope. Iodoform has no effect upon cultures until 5 per cent. is added. The fumes from four pounds of burning sulphur to each 1000 cubic feet of air space will kill tubercle bacilli in eight hours when fully exposed to the action of the gas, providing they are moist, or abundant moisture is present in the air. Formaldehyde gas is quicker in its action, but not much more efficient. Ten ounces of formalin should be employed for each 1000 cubic feet of air space. The tubercle bacillus resists the action of alkaline hypo-

unless the media be favorable, such as egg or serum; transplants after this time may fail to grow. A few bacilli, sufficient to infect guinea-pigs, may persist much longer. Cold has little effect upon them. When dry, some of the organisms stand dry heat at 100° C. for twenty minutes but are dead in forty-five minutes; but when in fluids and separated as in milk, they are quickly killed—viz., at 60° C. in twenty minutes, at 65° C. in fifteen minutes, at 70° C. the great majority in one minute, all in five minutes, at 80° C. the great majority in one-half minute, all in one minute, and at 95° C. in one-half minute. In some experiments they appear to withstand a higher temperature. As pointed out by Theobald Smith, when milk is heated in a test-tube in the usual way the cream which rises on heating is exposed on its surface to a lower temperature than the rest of the milk, and as this contains a large percentage of the bacteria some of them are exposed to less heat than those in the rest of the fluid. Rosenau points out another source of error: If a moderate number of killed bacilli are injected, limited lesions will arise and caseation may follow. On killing and autopsying the animals, tubercle bacilli can then be demonstrated in smears from the lesions, and the inoculation is considered positive. If, however, this material is reinjected into a second pig, the latter will show nothing on autopsy. This capacity of dead bacilli to cause macroscopic lesions has long been shown by Prudden and Hodenpyl. Its importance, however, is not sufficiently considered.

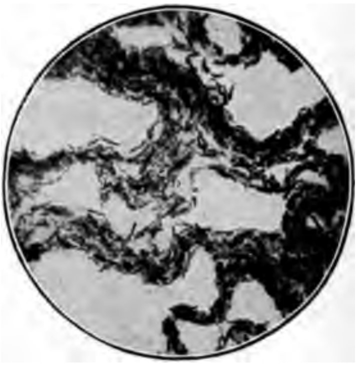

FIG. 142.—Tubercle bacilli. Impression preparation from small colony on coagulated blood serum. × 1000 diameters.

The resisting power of this bacillus to chemical disinfectants, drying, and light is considerable, but not as great as it is apt to appear, for, as in sputum, the bacillus is usually protected by mucus or cell protoplasm from penetration by the germicidal agent. It is not always destroyed by the gastric juice in the stomach, as is shown by successful infection experiments in susceptible animals by feeding them with tubercle bacilli. They are destroyed in sputum in six hours or less by the addition of an equal quantity of a 5 per cent. solution of carbolic acid. Bichloride of mercury is less suitable for the disinfection of sputum as it combines with the mucus and forms a more or less protecting envelope. Iodoform has no effect upon cultures until 5 per cent. is added. The fumes from four pounds of burning sulphur to each 1000 cubic feet of air space will kill tubercle bacilli in eight hours when fully exposed to the action of the gas, providing they are moist, or abundant moisture is present in the air. Formaldehyde gas is quicker in its action, but not much more efficient. Ten ounces of formalin should be employed for each 1000 cubic feet of air space. The tubercle bacillus resists the action of alkaline hypo-

chloride solution ("antiformin") in dilutions which quickly dissolve non-acidfast bacteria.

The tubercle bacillus in sputum when exposed to direct sunlight is killed in from a few minutes to several hours, according to the thickness of the layer and the season of the year; it is also usually destroyed by diffuse daylight in from five to ten days when placed near a window in fine powder. Protected in cloth the bacilli survive exposure to light for longer periods. Tuberculous sputum expectorated upon sidewalks, etc., when left undisturbed in the shade may be infectious for weeks, but when exposed to the action of direct sunlight will, in many cases, especially in summer, be disinfected by the time it is in condition to be carried into the air as dust, but not before children and flies have an opportunity of getting into it. The action of sunlight and other more important hygienic reasons suggest that the consumptive patients should occupy light, sunny rooms.

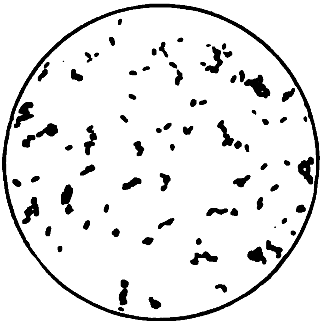

FIG. 143.—Tubercle bacilli, bovine. × 1000 diameters.

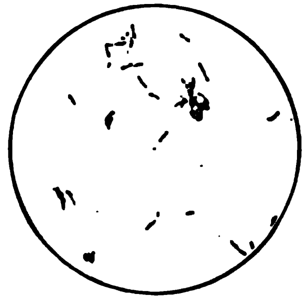

FIG. 144.—Tubercle bacilli, human. × 1000 diameters.

Dried sputum in rooms protected from abundant light has occasionally been found to contain virulent tubercle bacilli for as long as ten months. For a year at least it should be considered dangerous. The Röntgen rays have a deleterious effect on tubercle bacilli in cultures, but practically none upon those in tissues.

**Multiplication of Tubercle Bacilli in Nature Takes Place Only in the Living Animal.**—The tubercle bacillus is a strict parasite—that is to say, its biological characters are such that it could scarcely find natural conditions outside of the bodies of living animals favorable for its multiplication. Under exceptional conditions, such as in freshly expectorated sputum, tubercle bacilli may increase for a limited time.

**Cultivation of the Tubercle Bacillus.**—On account of their slow growth and the special conditions which they require, tubercle bacilli cannot be grown in pure culture by the usual plate method on ordinary culture media. Koch first succeeded in cultivating and isolating this bacillus on coagulated beef serum, which he inoculated by carefully rubbing the surface with sections of tuberculous tissue and then leaving the culture, protected from evaporation, for several weeks in the incubator. Cultures are more readily obtained of human or avian than of bovine bacilli.

**Growth on Coagulated Dog or Bovine Serum or on Egg.**—On these, one of which is generally used to obtain the first culture, the growth is usually visible at the end of ten days at 37° C., and at the end of three or four weeks a distinct and characteristic development has occurred. On serum small, grayish-white points and scales first appear on the surface of the medium. As development progresses there is formed an irregular, membranous-looking layer. On egg the growth is in the form of more or less elevated colonies which may become confluent.

**Growth on Nutrient 3 to 5 Per Cent. Glycerin Agar.**—Owing to the greater facility of preparing and sterilizing *glycerin agar*, it is now usually employed in preference to blood serum for continuing to produce later cultures. When numerous bacilli have been distributed over the surface of the culture medium, a rather uniform, thick, white layer, which subsequently acquires a slight yellowish tint, is developed; when the bacilli sown are few in number, or are associated in scattered groups, separate colonies are developed, which acquire considerable thickness and have more or less irregular outlines. The growth appears similar to that shown upon bouillon as seen in Fig. 145.

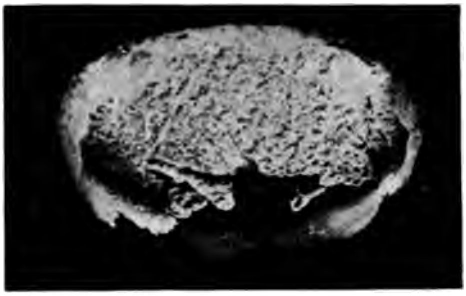

FIG. 145.—Growth of tubercle bacilli upon glycerin bouillon. (Kolle and Wasserman.)

**Growth on Nutrient Veal or Beef Broth Containing 5 Per Cent. of Glycerin.**—Glycerin broth is used for the development of tuberculin and must be neutral to litmus, viz., between 1.5 and 2 per cent. acid to phenol phthalein. On these media the tubercle bacillus grows readily if a very fresh thin film of growth from the glycerin agar or a small piece of pellicle removed from a previous broth culture is floated on the surface. This continues to enlarge as long as it floats on the surface of the liquid, and in the course of three to six weeks covers it wholly as a single film, which on agitation is easily broken up and then settles to the bottom of the flask, where it ceases to develop further. The liquid remains clear. A practical point of importance, if a quick growth is desired, is to use for the new cultures a portion of the pellicle of a growing bouillon culture, which is very thin and actively increasing.

**Growth on Potato.**—A good growth from cultures and sometimes even from tissue takes place on potato, and this forms the most uniform medium for stock cultures.

**Obtaining of Pure Cultures of the Tubercle Bacillus from Sputum, Infected Tissue, and Other Materials.**—On account of the time required and the difficulties to be overcome, this is never desirable except when careful investigations of importance are to be undertaken. *Pure cultures* can be obtained directly from tuberculous material, if the tubercle bacilli are present in sufficient number and mixed infection is not present, by using the proper blood serum or egg culture medium (p. 118); but it is difficult to get material free from other bacteria which grow much more rapidly and take possession of the medium before the tubercle bacillus has had time to form visible colonies. It is usually necessary first to inoculate guinea-pigs, subcutaneously or intramuscularly, preferably in the thigh, and then obtain cultures from the animals as soon as the tuberculous infection has developed. In this way, due to the susceptibility of the guinea-pig to tuberculosis, cultures may be obtained from material containing very few tubercle bacilli, although contaminating bacteria may be very numerous.

Animals inoculated usually die at the end of three weeks to four months. It is better, however, not to wait until the death of the animals, but at the end of four to six weeks to kill a guinea-pig without violence, using illuminating gas, chloroform, or ether in a closed tin or jar. (Animals which develop tuberculosis acutely are apt to have abundant tubercle bacilli and give successful cultures, while the chronic cases usually have few bacilli and may give unsuccessful cultures.) The animal after being killed is tied out in trays, and after washing with a 5 per cent. solution of carbolic acid, immediately autopsied. The skin over the anterior portion of the body having been carefully turned back, the inguinal nodes are removed with fresh instruments. The nodes on the side of injection are especially favorable for cultures. The abdomen is then opened and the spleen and retroperitoneal nodes removed. As the organs are removed they should be placed in Petri dishes and thoroughly minced with knife and forceps. Fresh instruments should be used for each operation. The sternal nodes may be used for cultures, but the lungs are almost useless, as the majority of cultures will be contaminated. The minced tissue is then placed on the surface of the culture media, both egg and glycerin egg being used, and evenly and thoroughly smeared over its surface, then the cotton plug is dipped in hot paraffin to aid in keeping the media from drying. The tubes are incubated in an inclined position. On egg, growth is visible in from seven to ten days, and well marked at the end of three weeks. Many tubes should be inoculated, as it is only with the dexterity acquired by practice that contaminations are avoided. As will be noted further on, the growth of the bovine type will be very sparse and on glycerin egg probably negative.

Cultures may also be obtained with a fair proportion of successful results by the antiformin method or the method of Petroff. In the former (see p. 434) the washed sediment is inoculated on egg media. Petroff (see p. 125) digests sputum or feces with equal amounts of 3 per cent. NaOH for one-half hour at 37° C., neutralizes with dilute HCl and inoculates the sediment obtained by centrifugalizing, on his special gentian-violet egg medium. A successful culture of an acid-fast bacillus does not necessarily mean a tubercle bacillus. Van Winkle in our laboratory has isolated two non-pathogenic acid-fast strains in this way from sputa.

**Pathogenesis.**—The tubercle bacillus is pathogenic not only for man, but for a large number of animals, such as the cow, monkey, pig, cat, etc. Young guinea-pigs are very susceptible, and are used for the detection of tubercle bacilli in suspected material. When inoculated with the minutest dose of the living bacilli they usually succumb to the disease. Infection is most rapidly produced by intraperitoneal injection. If a large dose is given, death follows in from ten to twenty days. The omentum is found to be clumped together in sausage-like masses which contain many bacilli. There is no serous fluid in the peritoneal cavity, but generally in both pleural sacs. The spleen is enlarged, and it, as well as the liver and peritoneum, contains large numbers of tubercle bacilli. If smaller doses are given, the disease is prolonged. The peritoneum and internal organs—spleen, liver, etc., and often the lungs—are then filled with tubercles. On subcutaneous injection, for instance, into the thigh, there is a thickening of the tissues about the point of inoculation, which may break down in one to three weeks and leave a sluggish ulcer covered with cheesy material. The neighboring lymph nodes are swollen, and at the end of two or three weeks may attain the size of hazel-nuts. Soon an irregular fever is set up, and the animal becomes emaciated, usually dying within four to eight weeks. If the injected material contained only a small number of bacilli the wound at the point of inoculation may heal up and death be postponed for a long time. At autopsy the lymphatic nodes are found to have undergone cheesy degeneration; the spleen may be very much enlarged, and throughout its substance, which is colored dark red, are distributed masses of nodules. The liver is also commonly increased in size, streaked brown and yellow, and the lungs are filled with grayish-white tubercles; but, as a rule, the kidneys contain no nodules. Occasionally the lesions are limited to the inguinal and retroperitoneal nodes. Tubercle bacilli are found in the affected tissues, but the more chronic the process the fewer the bacilli present.

Injection into the thigh is to be preferred for diagnostic purposes, the swelling of the local lymph nodes being then palpable. As soon as this is appreciable the node may be removed with or without killing the pig, the presence or absence of tuberculous lesions noted, and smears made for the detection of tubercle bacilli, thus saving time. It must be remembered that the pig may not show the usual picture of generalized tuberculosis, but only a swelling of the local lymph nodes. Fortunately tubercle bacilli are usually demonstrable in smears made from the crushed nodes. If there is any doubt the remaining tissue should be emulsified and reinjected into a second set of pigs. Another point to be considered is that other organisms may, rarely, give a picture difficult to distinguish macroscopically from tuberculosis, as, for instance, streptothrix. To safeguard against error smears should be stained and tubercle bacilli demonstrated. Chronic guinea-pig septicemia may be accompanied by lesions in the spleen which might be taken for tuberculous lesions.

Rabbits are very susceptible to tuberculosis of the bovine type, less so to that of the human type. This will be given more in detail under the differences between human and bovine tuberculosis.

Monkeys are very susceptible to infection with both types of bacilli. Cats, dogs, rats, and mice are susceptible, the last two usually show no tuberculous lesions, but there is great multiplication of the bacilli in the tissues.

**Tubercle Toxins.**—The tubercle bacillus produces no true toxins. The bodies of the bacteria contain substances which cause necrosis of tissue with subsequent caseation or abscess. In broth cultures, after filtration, are present substances which produce fever and inflammatory reactions of tissues. These substances as well as extracts from tubercle bacilli are highly toxic for tuberculous animals, little if at all for normal animals, and cause fever only in the former and the tissue inflammation spoken of as marked about tuberculous lesions. These poisons will be considered in detail later in connection with tuberculins.

**Action upon the Tissues of the Poisons Produced by the Tubercle Bacillus.**—Soon after the introduction into the tissues of tubercle bacilli, either living or dead, the cells surrounding them begin to show that some irritant is acting upon them. The connective-tissue cells become swollen and undergo mitotic division, the resultant cells being distinguished by their large size and pale nuclei. A small focus of proliferated epithelioid cells is thus formed about the bacilli, and according to the intensity of the inflammation these cells are surrounded by a larger or smaller number of lymphoid cells. When living bacilli are present and multiplying the lesions progress, the central cells degenerate and die, and a cheesy mass results, which later may lead to the formation of cavities. Dead bacilli, on the other hand, unless bunched together give off sufficient poison to cause the less marked changes only (Prudden and Hodenpyl). Of the gross pathological lesions produced in man by the tubercle bacilli the most characteristic are small nodules, called miliary tubercles. When young, and before they have undergone degeneration, these tubercles are gray and translucent in color, somewhat smaller than a millet-seed in size, and hard in consistence. But miliary tubercles are not the sole tuberculous products. The tubercle bacilli may cause diffuse growth of tissue identical in structure with that of miliary tubercles, that is, composed of a basement substance, containing epithelioid, giant, and lymphoid cells. This diffuse tuberculous tissue also tends to undergo cheesy degeneration.

**Point of Entrance of Infection.**—Infection by the tubercle bacillus takes place usually through the respiratory tract or the digestive tract, including the pharynx and tonsils, more rarely through wounds of the skin.

Tuberculosis may be considered to be caused chiefly by the direct transmission of tubercle bacilli to the mouth through soiled hands, lips, handkerchiefs, milk, etc., or by the inhalation of fine particles of mucus thrown off by coughing or loud speaking, or of dust contaminated by tuberculous sputum or feces.

**Tuberculosis of Skin and Mucous Membranes.**—When the skin or mucous membranes are superficially infected through wounds there may develop lupus, ulceration, or a nodular growth. The latter two forms of infection are apt after an interval to cause the involvement of the nearest lymphatic nodes.

**Tuberculosis of Respiratory Tract.**—The lungs are the most frequent location of clinically recognizable tuberculous inflammation. On account of their location they are greatly protected from external infection. Most of the bacilli are caught upon the nasal or pharyngeal mucous membranes. Only a small percentage can find their way to the larynx and trachea, and still less to the smaller bronchioles. From the examination of the lungs of miners as well as from experimental tests there is no doubt that some of the bacilli may find their way into the deeper bronchi. The deeper the bacilli penetrate the more unlikely that they can be cast out. On the other hand, the lungs are the most likely point of localization of tubercle bacilli which find their way into the blood stream. It is in this way that the lungs frequently become infected. It is now well established that infection taking place through the intestine may find its way by the blood to the lungs and excite there the most extensive lesions with or without leaving any trace of its point of entrance. Even if infection of the lung is slight or entirely absent the tubercle bacilli then find their way to the bronchial nodes where lesions may develop. Lesions either active or latent, anywhere in the body may be the source of a subsequent pulmonary infection. Should bacilli find their way into the blood, infection of the lung tissue will result if the resistance is lowered for any reason. The nasal cavities are rarely affected with tuberculosis, but more often the retropharyngeal tissue. Tuberculosis of this tissue as well as that of the tonsils is apt to give rise to infection of the lymph nodes of the neck. It is believed that just as bacilli may pass through the intestinal walls to infect the mesenteric nodes, so bacilli may, without leaving any trace, pass through the tonsils to the nodes of the neck.

Primary infection of the larynx is rare. Secondary infection is fairly common. The region of the vocal cords and the interarytenoid spaces are the special sites attacked.

**Infection by Inhalation of Dried and Moist Bacilli.**—A common mode of infection is by means of tuberculous sputum, which, being coughed up by consumptives, is either disseminated as a fine spray and so inhaled, or, carelessly expectorated, dried and, broken up by tramping over it, sweeping, etc., distributes numerous virulent bacilli in the dust. As long as the sputum remains moist there is no danger of infection, but only of direct contact; it is when it becomes dry, as on handkerchiefs, bedclothes, and the floor, etc., that the dust is a source of danger.

A great number of the expectorated and dried tubercle bacilli undoubt-

unless the media be favorable, such as egg or serum; transplants after this time may fail to grow. A few bacilli, sufficient to infect guinea-pigs, may persist much longer. Cold has little effect upon them. When dry, some of the organisms stand dry heat at 100° C. for twenty minutes but are dead in forty-five minutes; but when in fluids and separated as in milk, they are quickly killed—viz., at 60° C. in twenty minutes, at 65° C. in fifteen minutes, at 70° C. the great majority in one minute, all in five minutes, at 80° C. the great majority in one-half minute, all in one minute, and at 95° C. in one-half minute. In some experiments they appear to withstand a higher temperature. As pointed out by Theobald Smith, when milk is heated in a test-tube in the usual way the cream which rises on heating is exposed on its surface to a lower temperature than the rest of the milk, and as this contains a large percentage of the bacteria some of them are exposed to less heat than those in the rest of the fluid. Rosenau points out another source of error: If a moderate number of killed bacilli are injected, limited lesions will arise and caseation may follow. On killing and autopsying the animals, tubercle bacilli can then be demonstrated in smears from the lesions, and the inoculation is considered positive. If, however, this material is reinjected into a second pig, the latter will show nothing on autopsy. This capacity of dead bacilli to cause macroscopic lesions has long been shown by Prudden and Hodenpyl. Its importance, however, is not sufficiently considered.

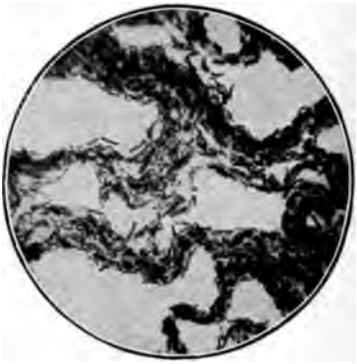

Fig. 142.—Tubercle bacilli. Impression preparation from small colony on coagulated blood serum. × 1000 diameters.

The resisting power of this bacillus to chemical disinfectants, drying, and light is considerable, but not as great as it is apt to appear, for, as in sputum, the bacillus is usually protected by mucus or cell protoplasm from penetration by the germicidal agent. It is not always destroyed by the gastric juice in the stomach, as is shown by successful infection experiments in susceptible animals by feeding them with tubercle bacilli. They are destroyed in sputum in six hours or less by the addition of an equal quantity of a 5 per cent. solution of carbolic acid. Bichloride of mercury is less suitable for the disinfection of sputum as it combines with the mucus and forms a more or less protecting envelope. Iodoform has no effect upon cultures until 5 per cent. is added. The fumes from four pounds of burning sulphur to each 1000 cubic feet of air space will kill tubercle bacilli in eight hours when fully exposed to the action of the gas, providing they are moist, or abundant moisture is present in the air. Formaldehyde gas is quicker in its action, but not much more efficient. Ten ounces of formalin should be employed for each 1000 cubic feet of air space. The tubercle bacillus resists the action of alkaline hypo-

chloride solution ("antiformin") in dilutions which quickly dissolve non-acidfast bacteria.

The tubercle bacillus in sputum when exposed to direct sunlight is killed in from a few minutes to several hours, according to the thickness of the layer and the season of the year; it is also usually destroyed by diffuse daylight in from five to ten days when placed near a window in fine powder. Protected in cloth the bacilli survive exposure to light for longer periods. Tuberculous sputum expectorated upon sidewalks, etc., when left undisturbed in the shade may be infectious for weeks, but when exposed to the action of direct sunlight will, in many cases, especially in summer, be disinfected by the time it is in condition to be carried into the air as dust, but not before children and flies have an opportunity of getting into it. The action of sunlight and other more important hygienic reasons suggest that the consumptive patients should occupy light, sunny rooms.

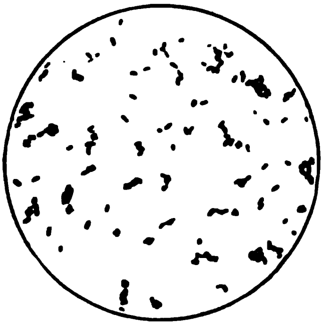

FIG. 143.—Tubercle bacilli, bovine. × 1000 diameters.

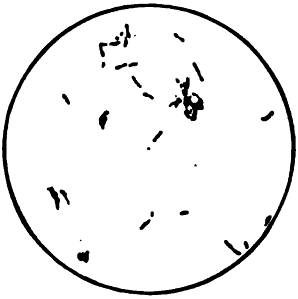

FIG. 144.—Tubercle bacilli, human. × 1000 diameters.

Dried sputum in rooms protected from abundant light has occasionally been found to contain virulent tubercle bacilli for as long as ten months. For a year at least it should be considered dangerous. The Röntgen rays have a deleterious effect on tubercle bacilli in cultures, but practically none upon those in tissues.

**Multiplication of Tubercle Bacilli in Nature Takes Place Only in the Living Animal.**—The tubercle bacillus is a strict parasite—that is to say, its biological characters are such that it could scarcely find natural conditions outside of the bodies of living animals favorable for its multiplication. Under exceptional conditions, such as in freshly expectorated sputum, tubercle bacilli may increase for a limited time.

**Cultivation of the Tubercle Bacillus.**—On account of their slow growth and the special conditions which they require, tubercle bacilli cannot be grown in pure culture by the usual plate method on ordinary culture media. Koch first succeeded in cultivating and isolating this bacillus on coagulated beef serum, which he inoculated by carefully rubbing the surface with sections of tuberculous tissue and then leaving the culture, protected from evaporation, for several weeks in the incubator. Cultures are more readily obtained of human or avian than of bovine bacilli.

**Growth on Coagulated Dog or Bovine Serum or on Egg.**—On these, one of which is generally used to obtain the first culture, the growth is usually visible at the end of ten days at 37° C., and at the end of three or four weeks a distinct and characteristic development has occurred. On serum small, grayish-white points and scales first appear on the surface of the medium. As development progresses there is formed an irregular, membranous-looking layer. On egg the growth is in the form of more or less elevated colonies which may become confluent.

**Growth on Nutrient 3 to 5 Per Cent. Glycerin Agar.**—Owing to the greater facility of preparing and sterilizing *glycerin agar,* it is now usually employed in preference to blood serum for continuing to produce later cultures. When numerous bacilli have been distributed over the surface of the culture medium, a rather uniform, thick, white layer, which subsequently acquires a slight yellowish tint, is developed; when the bacilli sown are few in number, or are associated in scattered groups, separate colonies are developed, which acquire considerable thickness and have more or less irregular outlines. The growth appears similar to that shown upon bouillon as seen in Fig. 145.

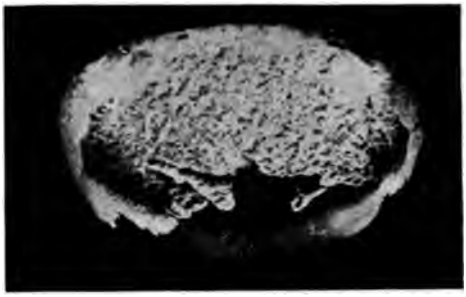

FIG. 145.—Growth of tubercle bacilli upon glycerin bouillon. (Kolle and Wasserman.)

**Growth on Nutrient Veal or Beef Broth Containing 5 Per Cent. of Glycerin.**—Glycerin broth is used for the development of tuberculin and must be neutral to litmus, viz., between 1.5 and 2 per cent. acid to phenol phthalein. On these media the tubercle bacillus grows readily if a very fresh thin film of growth from the glycerin agar or a small piece of pellicle removed from a previous broth culture is floated on the surface. This continues to enlarge as long as it floats on the surface of the liquid, and in the course of three to six weeks covers it wholly as a single film, which on agitation is easily broken up and then settles to the bottom of the flask, where it ceases to develop further. The liquid remains clear. A practical point of importance, if a quick growth is desired, is to use for the new cultures a portion of the pellicle of a growing bouillon culture, which is very thin and actively increasing.

**Growth on Potato.**—A good growth from cultures and sometimes even from tissue takes place on potato, and this forms the most uniform medium for stock cultures.

**Obtaining of Pure Cultures of the Tubercle Bacillus from Sputum, Infected Tissue, and Other Materials.**—On account of the time required and the difficulties to be overcome, this is never desirable except when careful investigations of importance are to be undertaken. *Pure cultures* can be obtained directly from tuberculous material, if the tubercle bacilli are present in sufficient number and mixed infection is not present, by using the proper blood serum or egg culture medium (p. 118); but it is difficult to get material free from other bacteria which grow much more rapidly and take possession of the medium before the tubercle bacillus has had time to form visible colonies. It is usually necessary first to inoculate guinea-pigs, subcutaneously or intramuscularly, preferably in the thigh, and then obtain cultures from the animals as soon as the tuberculous infection has developed. In this way, due to the susceptibility of the guinea-pig to tuberculosis, cultures may be obtained from material containing very few tubercle bacilli, although contaminating bacteria may be very numerous.

Animals inoculated usually die at the end of three weeks to four months. It is better, however, not to wait until the death of the animals, but at the end of four to six weeks to kill a guinea-pig without violence, using illuminating gas, chloroform, or ether in a closed tin or jar. (Animals which develop tuberculosis acutely are apt to have abundant tubercle bacilli and give successful cultures, while the chronic cases usually have few bacilli and may give unsuccessful cultures.) The animal after being killed is tied out in trays, and after washing with a 5 per cent. solution of carbolic acid, immediately autopsied. The skin over the anterior portion of the body having been carefully turned back, the inguinal nodes are removed with fresh instruments. The nodes on the side of injection are especially favorable for cultures. The abdomen is then opened and the spleen and retroperitoneal nodes removed. As the organs are removed they should be placed in Petri dishes and thoroughly minced with knife and forceps. Fresh instruments should be used for each operation. The sternal nodes may be used for cultures, but the lungs are almost useless, as the majority of cultures will be contaminated. The minced tissue is then placed on the surface of the culture media, both egg and glycerin egg being used, and evenly and thoroughly smeared over its surface, then the cotton plug is dipped in hot paraffin to aid in keeping the media from drying. The tubes are incubated in an inclined position. On egg, growth is visible in from seven to ten days, and well marked at the end of three weeks. Many tubes should be inoculated, as it is only with the dexterity acquired by practice that contaminations are avoided. As will be noted further on, the growth of the bovine type will be very sparse and on glycerin egg probably negative.

Cultures may also be obtained with a fair proportion of successful results by the antiformin method or the method of Petroff. In the former (see p. 434) the washed sediment is inoculated on egg media. Petroff (see p. 125) digests sputum or feces with equal amounts of 3 per cent. NaOH for one-half hour at 37° C., neutralizes with dilute HCl and inoculates the sediment obtained by centrifugalizing, on his special gentian-violet egg medium. A successful culture of an acid-fast bacillus does not necessarily mean a tubercle bacillus. Van Winkle in our laboratory has isolated two non-pathogenic acid-fast strains in this way from sputa.

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**Action upon the Tissues of the Poisons Produced by the Tubercle Bacillus.**—Soon after the introduction into the tissues of tubercle bacilli, either living or dead, the cells surrounding them begin to show that some irritant is acting upon them. The connective-tissue cells become swollen and undergo mitotic division, the resultant cells being distinguished by their large size and pale nuclei. A small focus of proliferated epithelioid cells is thus formed about the bacilli, and according to the intensity of the inflammation these cells are surrounded by a larger or smaller number of lymphoid cells. When living bacilli are present and multiplying the lesions progress, the central cells degenerate and die, and a cheesy mass results, which later may lead to the formation of cavities. Dead bacilli, on the other hand, unless bunched together give off sufficient poison to cause the less marked changes only (Prudden and Hodenpyl). Of the gross pathological lesions produced in man by the tubercle bacilli the most characteristic are small nodules, called miliary tubercles. When young, and before they have undergone degeneration, these tubercles are gray and translucent in color, somewhat smaller than a millet-seed in size, and hard in consistence. But miliary tubercles are not the sole tuberculous products. The tubercle bacilli may cause diffuse growth of tissue identical in structure with that of miliary tubercles, that is, composed of a basement substance, containing epithelioid, giant, and lymphoid cells. This diffuse tuberculous tissue also tends to undergo cheesy degeneration.

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**Tuberculosis of Respiratory Tract.**—The lungs are the most frequent location of clinically recognizable tuberculous inflammation. On account of their location they are greatly protected from external infection. Most of the bacilli are caught upon the nasal or pharyngeal mucous membranes. Only a small percentage can find their way to the larynx and trachea, and still less to the smaller bronchioles. From the examination of the lungs of miners as well as from experimental tests there is no doubt that some of the bacilli may find their way into the deeper bronchi. The deeper the bacilli penetrate the more unlikely that they can be cast out. On the other hand, the lungs are the most likely point of localization of tubercle bacilli which find their way into the blood stream. It is in this way that the lungs frequently become infected. It is now well established that infection taking place through the intestine may find its way by the blood to the lungs and excite there the most extensive lesions with or without leaving any trace of its point of entrance. Even if infection of the lung is slight or entirely absent the tubercle bacilli then find their way to the bronchial nodes where lesions may develop. Lesions either active or latent, anywhere in the body may be the source of a subsequent pulmonary infection. Should bacilli find their way into the blood, infection of the lung tissue will result if the resistance is lowered for any reason. The nasal cavities are rarely affected with tuberculosis, but more often the retropharyngeal tissue. Tuberculosis of this tissue as well as that of the tonsils is apt to give rise to infection of the lymph nodes of the neck. It is believed that just as bacilli may pass through the intestinal walls to infect the mesenteric nodes, so bacilli may, without leaving any trace, pass through the tonsils to the nodes of the neck.

Primary infection of the larynx is rare. Secondary infection is fairly common. The region of the vocal cords and the interarytenoid spaces are the special sites attacked.

**Infection by Inhalation of Dried and Moist Bacilli.**—A common mode of infection is by means of tuberculous sputum, which, being coughed up by consumptives, is either disseminated as a fine spray and so inhaled, or, carelessly expectorated, dried and, broken up by tramping over it, sweeping, etc., distributes numerous virulent bacilli in the dust. As long as the sputum remains moist there is no danger of infection, but only of direct contact; it is when it becomes dry, as on handkerchiefs, bedclothes, and the floor, etc., that the dust is a source of danger.

A great number of the expectorated and dried tubercle bacilli undoubtonly be destroyed, as we have said, when acted upon by direct sunlight, but when it is considered that as many as five billion virulent tubercle bacilli may be expectorated by a single tuberculous individual in twenty-four hours it is evident that even a much smaller proportion than are known to stay alive will suffice in the immediate vicinity of consumptives to produce infection unless precautions are taken to prevent it. The danger of infection is greatest, of course, in the close neighborhood of tuberculous patients who expectorate profusely and indiscriminately, that is, without taking the necessary means for preventing infection. We found that of 100 tuberculous men admitted to one of the consumption hospitals, only 20 claimed to have taken any care to prevent the contamination of their surroundings by their sputum. There is much less danger of infection at a distance, as in the streets for instance, where the tubercle bacilli have become so diluted that they are less to be feared. In rooms the sputum is not only protected from the direct sunlight, but it is constantly broken up and blown about by the walking, closing of doors, etc. In crowded streets on windy days infected dust must sometimes be in the air unless the expectoration of consumptives is controlled.

Exhaustive experiments made by many observers have shown that particles of dust collected from the immediate neighborhood of consumptives, when inoculated into guinea-pigs, produce tuberculosis in a considerable percentage of them; whereas, the dust from rooms inhabited by healthy persons or dust of the streets does so only in an extremely small percentage. Flügge is probably right in thinking that the dust which is fine enough to remain for a long time in suspension in the air is usually free from living bacilli. It is in the coarser though still minute particles, those in which the bacilli are protected by an envelope of mucus, that the germs resist drying for considerable periods. These are carried only short distances by air currents. Such reports are those given by Straus, who, on examining the nasal secretions of twenty-nine healthy persons living in a hospital with consumptive patients, found tubercle bacilli in nine of them, must be accepted with some reserve, since we know that in the air there are bacilli which look and stain like tubercle bacilli and yet are totally different. It may be said that the danger of infection from slight contact with the tuberculous is not so great as it is considered by many, but that on this account it is all the more to be guarded against in the immediate neighborhood of consumptives. Those who are most liable to infection from this source are young children; the adult members of the family, the nurses, the fellow-workmen, and fellow-prisoners of persons suffering from the disease are relatively very less likely to become infected. In this connection, also, attention may be drawn to the fact that rooms which have been recently occupied by consumptives are not infrequently the means of producing infection (as has been clinically and experimentally demonstrated) from the deposition of tuberculous dust on furniture, walls, floors, etc. The danger is not apt to last beyond three months. Flügge has drawn attention to the fact that in coughing sneezing, etc., very fine particles of throat secretion containing bacilli are thrown out and carried by air currents many feet from the patient and remain suspended in the air for a considerable time. To encourage us, however, we now have a mass of facts which go to show that when the sputum is carefully looked after there is very little danger of infecting others except by close personal contact.

**Tuberculosis of Digestive Tract.**—Tuberculosis of the gums, cheeks, or tongue is rare. The tonsils and pharynx are somewhat more often involved. The stomach and esophagus are almost never attacked. The small intestines are rather frequently the seat of infection from bacilli swallowed with the food or dust-infected mucus. In a striking case four previously healthy children died within a short period of one another. Their nurse was found to have tuberculosis of the antrum of Highmore, with a fistulous opening into the mouth. She had the habit of putting the spoon with which she fed the children into her mouth so as to taste the food before it was given to them.

As already noted, the bacilli frequently pass through the mucous membrane of the gastro-intestinal canal to the lymph glands without leaving any lesions.

**Infection by Ingestion of Milk and Milk Products.**—Milk serves as a conveyer of infection, whether it be the milk of tuberculous mothers or the milk of tuberculous cows. In this case evidence of infection is usually shown in the mesenteric and cervical lymph nodes or generalized tuberculosis may be caused, while the intestinal walls are frequently not affected. Bacilli accompanied by fat pass much more readily through the intestinal mucous membrane or that of the tonsils and pharynx. The transmission of tubercle bacilli by the milk of tuberculous cows has been abundantly proved.

Formerly it was thought that in order to produce infection by milk there must be a local tuberculous affection of the udder; but it is now known that tubercle bacilli may be found in the milk in small numbers, when adjacent tissue is infected and when careful search fails to detect any udder disease. Schröder has shown that the feces are a very dangerous factor in the dissemination of tubercle bacilli. He compares feces in cattle to sputum in man, since the tubercle bacilli are swallowed by cattle and are to a great extent passed through the intestinal tract without destruction. He found that when milk from phthisical cows having healthy udders was obtained so as not to become infected by feces it was free from bacilli, but when obtained without special precautions it was frequently infected. The milk of every cow which has any well-developed internal tuberculous infection must therefore be considered as possibly containing tubercle bacilli. Rabinowitsch, Kempner, and Mohler also proved beyond question that not only the milk of tuberculous cattle, which showed no appreciable udder disease, but also those in which tuberculosis was only detected through tuberculin, frequently contained tubercle bacilli. Different observers have found tubercle bacilli in 10 to 30 per cent. of the samples of unheated city milk. Butter may contain tubercle bacilli in higher percentages of samples examined. When we consider the prevalence of tuberculosis among cattle we can readily realize that, even if the bovine bacillus infects human beings with difficulty, there is danger to children when they are exposed to this source of infection. The milk from cattle suffering from udder tuberculosis usually contains a few hundred bacilli per cubic centimeter, but may contain many millions. It is also important to mention the fact that mixed milk from a herd, though tending to dilute the milk of cows excreting tubercle bacilli, may be badly infected from one cow, especially if this cow has udder disease.

Taking the abattoir statistics of various countries, we find that about 10 per cent. of the cattle slaughtered were tuberculous. A less probable source of infection by way of the intestines is the flesh of tuberculous cattle. Here the danger is considerably less, from the fact that meat is usually cooked, and also because the muscular tissues are seldom attacked. In view of the finding of the bovine type of bacilli in a considerable percentage of the cases of tuberculous children tested, the legislative control and inspection of cattle and milk is an absolute necessity. As a practical and simple method of preventing infection from suspected milk, sufficient heating of the milk used as food must commend itself to all. Human tubercle bacilli may be found in milk as instanced by one sample out of a series of city milks examined in the Research Laboratory by Hess.

**Method of Examining Milk for Tubercle Bacilli.**—Thirty cubic centimeters of milk are centrifuged at high speed and 10 c.c. of the lower milk and sediment collected. Four cubic centimeters of the cream is thinned with a little sterile water and injected into two guinea-pigs. The sediment is injected in amounts of 3 to 5 c.c. into other pigs. Larger amounts than this are apt to kill too many pigs from the associated bacteria. Subcutaneous injection is to be preferred. There are certain precautions that must be taken in drawing conclusions, as the different types of acid-fast "butter bacilli" may cause lesions, and their presence will be noted in smears made from these lesions. To avoid this source of error, two methods are resorted to. If cultures are made from the suspected lesions on glycerin agar, these bacilli usually develop in a few days, whereas tubercle bacilli do not. When one is ready to kill the pigs, 2 c.c. of old tuberculin should be injected into each pig late in the day. The following morning the tuberculous pigs will be dead or dying. Autopsies should be done on all to confirm the test. The milk should be as fresh as possible to prevent the growth of other bacteria.

**Bovine Infection in Man.**—Numerous investigations have been made on this point. To Ravenel probably belongs the credit of isolating the first bovine bacillus from a child. The following tables summarizing the results of a large series of cases give a fair idea of incidence of such infection. As will be seen, children are especially the ones infected, and usually the point of entry is clearly alimentary, as shown by the lesions. Cervical adenitis and abdominal tuberculosis are the most frequent types of infection. Generalized tuberculosis due to bovine infection is less frequent. Bone and joint tuberculosis is usually of the human type. The meninges are less commonly affected by the bovine type than by the human type. Infection of adults is very uncommon; and, though cases of pulmonary tuberculosis due to the bovine type of bacillus have been reported, such cases are rare.

TABLE I.—TABULATION OF CASES REPORTED.[1]

| Diagnosis. | Adults sixteen years and over. | | Children five to sixteen years. | | Children under five years. | |
|---|---|---|---|---|---|---|
| | Human | Bovine. | Human. | Bovine. | Human. | Bovine. |
| Pulmonary tuberculosis | 497 | 3 | 6 | — | 28 | 1 |
| Tuberculous adenitis, axillary | 2 | — | — | — | 2 | — |
| Tuberculous adenitis, cervical | 27 | 1 | 17 | 14 | 9 | 11 |
| Abdominal tuberculosis | 15 | 4 | 7 | 8 | 9 | 11 |
| Generalized tuberculosis, alimentary origin | 6 | 1 | 3 | 4 | 16 | 13 |
| Generalized tuberculosis | 27 | — | 4 | 1 | 56 | 3 |
| Generalized tuberculosis, including meninges, alimentary origin | — | — | 1 | — | 5 | 10 |
| Generalized tuberculosis, including meninges | 4 | — | 10 | — | 51 | — |
| Tubercular meningitis | — | — | 1 | — | 2 | 2 |
| Tuberculosis of bones and joints | 31 | 1 | 31 | 3 | 20 | — |
| Genito-urinary tuberculosis | 16 | — | 1 | — | — | — |
| Tuberculosis of skin | 9 | 3 | 4 | 6 | 2 | — |
| Miscellaneous cases: | | | | | | |
| Tuberculosis of tonsils | — | — | — | 1 | — | — |
| Tuberculosis of mouth and cervical nodes | — | 1 | — | — | — | — |
| Tuberculous sinus or abscess | 1 | — | — | — | — | — |
| Sepsis, latent bacilli | — | — | — | — | 1 | — |
| Totals | 635 | 14 | 85 | 37 | 201 | 51 |

| | | | |
|---|---|---|---|
| Mixed or double infections: | 10 | cases. | |
| Pulmonary tuberculosis, | 20 | years. | Sputum, human and bovine types. |
| | 27 | years. | Sputum, human and bovine types. |
| Abdominal tuberculosis, | 70 | years. | Mesenteric nodes, human type.<br>Retroperitoneal, human and bovine types. |
| Generalized tuberculosis (alimentary origin), | 5¾ | years. | Spleen, human type.<br>Mesenteric node, bovine type. |
| | 18 | years. | Lung, culture not obtained.<br>Mesenteric nodes, human and bovine types. |
| | 30 | years. | Bronchial node, human type.<br>Mesenteric nodes, human and bovine types. |
| Generalized tuberculosis, | 9 | months. | Bronchial nodes, bovine type.<br>Mesenteric nodes, human type. |
| Generalized tuberculosis, including meninges (alimentary origin). | 4 | years. | Meninges, human type.<br>Bronchial nodes, human type.<br>Mesenteric nodes, bovine type. |
| Generalized tuberculosis, including meninges. | 4 | years. | Bronchial nodes } injected together.<br>Spleen } bovine and human types. |
| | $4\frac{7}{12}$ | years. | Meninges, human type.<br>Lung, human type.<br>Mesenteric nodes, human type.<br>Bronchial nodes, human and bovine types. |

Total cases, 1033.

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[1] Summary of cases reported up to July, 1912, exclusive of cases examined at the Research Laboratory (see Table II). In contrast to the next table, the above contains a large percentage of selected cases of alimentary types of tuberculosis, many of which showed only slight lesions.

TABLE II.—THE RELATIVE PROPORTION OF HUMAN AND BOVINE TUBERCLE BACILLI INFECTIONS IN A LARGE SERIES OF UNSELECTED CASES[1] EXAMINED AT THE RESEARCH LABORATORY.

| Diagnosis of cases examined. | Adults sixteen years and over. | | Children five to sixteen years. | | Children under five years. | | Notes. |
|---|---|---|---|---|---|---|---|
| | Human. | Bovine. | Human. | Bovine. | Human. | Bovine. | |
| Pulmonary tuberculosis | 281 | – | 8 | – | 7 | – | Clinical diagnosis only known and therefore no positive details as to the extent of lesions elsewhere. |
| Tuberculous adenitis, inguinal and axillary | 1 | – | 4 | – | – | – | See next. |
| Tuberculous adenitis, cervical | 9 | – | 19 | 8 | 6 | 13 | In two cases cultures were from axillary nodes but the primary focus was cervical. Another case died shortly afterward with pulmonary tuberculosis. |
| Abdominal tuberculosis | 1 | – | 1 | 1 | 1 | 3 | Milk supply of one child subsequently examined. Tubercle bacilli isolated. |
| Generalized tuberculosis, alimentary origin | – | – | – | – | 1 | 2 | Only three cases given under this heading. Many of the cases in the following subdivisions showed marked intestinal lesions and some possibly were of alimentary origin. |
| Generalized tuberculosis | 2 | – | 1 | – | 18 | 4 | One bovine case had tuberculous osteomyelitis of the metatarsal bone. |
| Generalized tuberculosis including meninges | 1 | – | – | – | 25 | 1 | |
| Tuberculous meningitis | 1 | – | 2 | – | 26 | 2 | No autopsy. Extent of lesions elsewhere unknown. |
| Tuberculosis of bones and joints | 1 | – | 10 | – | 7 | – | |
| Genito-urinary tuberculosis | 6 | 1 | 1 | – | – | – | The adult bovine case was tuberculosis of kidney. Removal of kidney. Complete recovery. |
| Tuberculosis of skin | 1 | – | – | – | – | – | |
| Tuberculous abscess | 1 | – | – | – | – | – | Possibly primary in bone. |
| Totals | 305 | 1 | 46 | 9 | 91 | 25 | |

Double infection in one case. Both types isolated. Generalized tuberculosis including meninges, thirteen months. Mesenteric nodes gave human type. Meningeal fluid gave bovine type.

Total cases, 478.

A careful study of all the factors leads us to estimate that with the average raw milk supply about 10 per cent. of all deaths caused by tuberculosis in children under five is due to bovine infection.

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[1] Unselected cases from the hospitals of New York City. For full résumé and discussion of results see Park and Krumwiede, Jour. Med. Res., vols., xxiii, xxv and xxvii.

The tables on pages 423 and 424 give a summary of the results obtained in the larger investigations so far carried out.

**Hypothesis of Transmissibility of Tubercle Bacilli to the Fetus.**—The transmission of tubercle bacilli from the mother to the fetus in animals occurs occasionally. With regard to tuberculosis in the human fetus the evidence is not so clear, though some 20 cases have been recorded of tuberculosis in newly born infants, and about a dozen cases of placental tuberculosis. As to the infection of the fetus from the paternal side, where the father has tuberculosis of the scrotum or seminal vessels, we have no reason to suppose that such can occur. There are, however, grounds for belief that infection in this way may take place from husband to wife.

**Attenuation.**—Tubercle bacilli when subjected to deleterious influences slowly decrease in virulence. Some strains lose their virulence when artificially cultivated for some time; some quickly, some slowly. Others retain their virulence indefinitely.

**Mixed Infection.**—In regions where tuberculous processes are on the surface, such as skin infections, and also when the infection itself is multiple, as in diseases of the glands of the neck from tonsillar absorption, there are frequently associated with the tubercle bacilli one or more other varieties of organisms. Those of most importance are the streptococcus, pneumococcus and influenza bacilli. While the influence of this secondary or mixed infection is not exactly known, yet both the local and systemic effects are undoubtedly unfavorable.

In regard to pulmonary tuberculosis, it should be remembered that Baldwin has shown that caseation, ulceration and cavity formation may be produced experimentally in the lungs of animals by the tubercle bacillus alone. Further, it has been found that fever, emaciation and other characteristic symptoms of tuberculosis may be caused by the tubercle bacillus independently of any other associated microörganisms. The preponderance of the evidence supports the view that the lesions and symptoms in this type of infection may be caused by the tubercle bacillus alone, but not infrequently secondary organisms may contribute to the more severe symptoms or may be largely responsible for the unfavorable progress of the disease.

**Individual Susceptibility.**—It was believed by many that in demonstrating that tuberculosis was due to a specific bacillus its occurrence was sufficiently explained; but they left out another important factor in the production of disease—individual susceptibility. That this susceptibility, or "predisposition," as it is improperly called, may be either inherited or acquired is now an accepted fact in medicine. It has even been thought that the physical signs and characters—the *phthisical habit*—which indicate this susceptibility can be externally recognized. At first the inherited susceptibility was considered more important than the acquired, but now much that was attributed to the former is known to be explained by the fact of living in an infected area. The acquired susceptibility may arise from faulty physical development or from depression, sickness, overwork, excessive use of alcohol, etc.

Unquestionably, vast differences exist in different individuals in the intensity of the tuberculous process in the lung. That this does not depend chiefly upon a difference in virulence of the infection is evident from the fact that individuals contracting tuberculosis from the same source are attacked with different severity, and that there is, as a rule, no great difference in degrees of virulence for animals in the tubercle bacilli obtained from different sources. The possibility of favorably influencing many cases of an existing tuberculosis by treatment also proves that, under natural conditions, there is a varying susceptibility to the disease. Clinical experience teaches, likewise, that good hygienic conditions, pure air, good food, freedom from care, etc., increase resistance and are aids to recovery. Animal experiments have shown that not only are there differences of susceptibility in various animal species, but also an individual susceptibility in the same species. The doctrine of individual susceptibility, therefore, is seen to be founded on fact, although the reasons for it are only partially understood. Certain infectious diseases reduce the resistance to tuberculous infection, the most noteworthy example being measles.

**Tuberculosis Immunity.**—As in other infectious diseases, various attempts have been made to produce an artificial immunity against tuberculosis, but the results so far have been disappointing. The expectation that the immunological mechanism operating in other bacterial diseases would also be found in tuberculosis has not been realized. As a consequence of recent researches, we are coming to the opinion that the usual manifestations of humoral immunity have a secondary importance in this disease and that to the tuberculous focus we must ascribe a greater role than has been given it in the past. Tuberculous infection presents certain phases which find no close analogy in other infectious processes. Several facts of fundamental importance have developed from the newer pathological and bacteriological investigations. An infection by the tubercle bacillus may take place, and foci of considerable size may develop without the production of sufficient disturbance to the bodily welfare to attract attention. Further, while a frank tuberculous infection may come to a clinical cure, yet the focus remains, and, even though completely walled off, may harbor virulent tubercle bacilli during the whole of the individual's normal life. It has now become established that a healed tuberculous focus present in the body, containing as it does bacillary protein, exerts an appreciable influence on the behavior of the body to subsequent infection. In the consideration of tuberculosis immunity, therefore, we must carefully bear in mind the distinction between tuberculous infection and tuberculous disease.

Koch was the first to discover that animals already infected with living bacilli reacted differently to an injection of tubercle bacilli than did normal animals. When virulent tubercle bacilli are injected into a healthy animal, tubercles develop at or near the point of inoculation and the infection then progresses and the tubercle bacilli are carried to the spleen, liver, lungs and the intermediate glands with the formation of foci in these various organs. As a consequence, the animal finally succumbs to generalized tuberculosis. In the case, however, of an animal already tuberculous, the inoculation with virulent tubercle bacilli is followed by a quite different sequence of events. Shortly after such an inoculation there is a marked inflammatory reaction at the point of injection, followed by necrosis and possibly sloughing but with no advance of the infection beyond the point of inoculation. In other words, the animal suffers from a local toxic process but not from a true infection. Römer has given further details of this phenomenon. He found that if a small dose of tubercle bacilli be given at the second inoculation the local reaction soon subsides and healing results. But, on the other hand, if a large quantity of tubercle bacilli be injected the local process goes on to necrotic sloughing and the animal soon dies of cachexia. The tuberculous animal, therefore, when subjected to an injection of tubercle bacilli is not truly infected but suffers from an intoxication, the degree of which depends upon the quantity of tubercle bacilli introduced. Recent studies in tuberculosis emphasize the fundamental importance of Koch's and Römer's observations, and have forced a radical revision in our theories of tuberculous infection and immunity. By means of the various tuberculin reactions, particularly the intracutaneous of Mantoux and cutaneous of von Pirquet, and by the most careful postmortem studies on a great number of individuals, we now know that a large majority of human beings are infected with the tubercle bacillus and develop demonstrable tuberculous lesions before they reach the age of eighteen. A few children thus infected may succumb to the disease but by far the greater number show no conspicuous signs of the infection, their foci heal, and without the tuberculin reaction or the opportunity of examination after death, the presence of tuberculous infection would pass unsuspected and undetected.

These recent additions to our knowledge have given us a new and apparently a truer conception of the factors underlying tuberculous infection. It is quite probable that the majority of cases of pulmonary tuberculosis developing after the eighteenth year of age are caused not by infection from without but by the breaking down of an encapsulated focus acquired and healed during childhood. The greater morbidity during the age group of about twenty to thirty may be accounted for by the fact that this is usually the period of greatest physical and mental strain and stress and that it is this stress which breaks down the body's resistance to an infective focus, and which transforms a latent infection into active disease.

Although it is evident that the presence of a healed focus protects the individual to a certain degree against a subsequent infection by the tubercle bacillus, it is equally apparent that the exacerbations suffered by healed cases show that the protection acquired is at best only a relative one. We must therefore conclude that there exists in the human race no absolute immunity to tuberculosis, and that such increased resistance as we may possess is gained only at the hazard of an early infection.

**Immunization.**—Koch, reasoning from his observations on the ability of the tuberculous animal to resist subsequent infection, attempted to increase this resistance by the injection of certain modified products of the tubercle bacillus. To this end he prepared his original tuberculin, or "O. T." which, however, failed to fulfil his expectations. With the development of our modern theories of bacterial immunity the hope arose that protection might be actively acquired through the injection of the tubercle bacillus either attenuated or dead or of its products by way of stimulating the body to the production of specific antibodies; or, again, that protection might be passively conferred by the administration of the serum of animals made immune to the tubercle bacillus. From observations on experimental animals and from serological tests on individuals treated with the various tuberculins, we now know that the injection of antigens derived from the tubercle bacillus fails to call forth in the treated individual any marked response in the way of the production of demonstrable bodies and that serum therapy is of little or no avail.

It is true that in some cases resistance to the disease can be raised within certain limits, and that, in man, clinical cures may sometimes be effected by the aid of such therapeutic agents as tuberculin. Yet in these cases we find little or no evidence of the production of agglutinins, precipitins, lysins or complement-fixing antibodies. We are, consequently, forced to the conclusion that such immunity as has been established must have been the result of a physiological mechanism differing from the processes operating in other infectious diseases, and recent investigations, particularly those of Krause, point to the tuberculous focus as the prime factor in the production of such immunity as develops in tuberculous disease. Experimental analyses of the physiological action of tuberculin have shed new light on the problem. That it is the tuberculous focus which, in a large measure, determines the body's reactivity to tuberculin is shown by the fact that normal individuals, that is, individuals free from tuberculous infection, can tolerate the administration of a relatively large amount of tuberculin without exhibiting any appreciable symptoms. Tuberculin, therefore, is in itself non-toxic and the characterististic reaction following its application to the tuberculous body must be looked upon as an allergic phenomenon. Its manifestations are threefold: there is a local reaction at the point of application, a focal reaction at the site of infection and a general constitutional reaction. The local reaction appears as a more or less non-infective inflammation in the skin or on the mucous membranes, depending upon the point of application, and it is this phase of the reaction which has been so profitably utilized for the diagnosis of tuberculous infection (see "Tuberculin as a Diagnostic Aid," below). The focal reaction consists in vascular changes at the tuberculous lesion. There is a hyperemia with a consequent softening of the focus, and a liberation of antigenic or toxic focal products. Krause has shown that these products are inherently toxic and their liberation into the blood stream may occasion the fever, the malaise and the other symptoms characteristic of tuberculin intoxication. It is likely that in addition to this the antigen of the tuberculin may react with such immune bodies as may be present in the infected, and therefore hypersensitive, body and thus contribute to the general reaction.

If the dose of tuberculin is not too great the hyperemia at the focus is transient and the body responds with an increased cellular activity at the focus, resulting, under favorable conditions, in a further proliferation of connective tissue and a more complete encapsulation of the tuberculous lesion. Should the dose of tuberculin, on the other hand, be excessive or the bodily resistance be deficient the focal reaction may lead to a softening and breaking down of the lesion with consequent bleeding, dissemination of the liberated tubercle bacilli and extension of the lesion. Inasmuch as the internal location of the lesion frequently precludes an opportunity for observing the focal reaction we must look to the general constitutional manifestations as an index of the degree of reaction produced. The goal of tuberculin treatment, therefore, is the stimulation of the focus by doses of tuberculin so graded and adjusted to the sensitiveness of the individual that the body's ability to respond to and control the focal hyperemia is not overtaxed. In this way cell proliferation, encapsulation and ultimate healing of the focus may be promoted.

**Tuberculin in Diagnosis and Therapy.**—From the foregoing it can be readily seen that by taking advantage of the allergic or hypersensitive state in tuberculosis we are able to use tuberculin both as a diagnostic aid and as a therapeutic agent. *Tuberculin should never be sprayed out of a syringe. It may cause serious reactions in those exposed.*

**As a Diagnostic Aid.**—The presence of a tuberculous focus in the body, by some mechanism not yet wholly understood, sensitizes the skin and mucous membranes in such a way that the application of tuberculin to these tissues causes a more or less severe inflammatory reaction at the point of application. The preparation used is the Old Tuberculin ("O. T.") of Koch and the following represents the methods of application in their order of sensitiveness as described by Hamman.

1. Intracutaneous test of Mantoux.
2. Subcutaneous-local test ("Stich Reaktion").
3. Cutaneous test of von Pirquet.
4. Subcutaneous test.
5. Percutaneous test of Moro.
6. Conjunctival test of Calmette.

**The Intracutaneous Test of Mantoux.**—While more difficult to carry out than the cutaneous test, the intracutaneous is the more delicate, and is the most widely used. The test is carried out as follows: the inner surface of the forearm is cleansed with alcohol, then with ether, the skin is drawn taut, and the diluted tuberculin is injected from a tuberculin syringe (1 c.c. graduated in 50ths or 100ths) through a fine needle (a No. 26 preferably) which has been carefully inserted into, but not under, the skin. The total volume injected should be 0.1 c.c., and it is advisable to employ several dilutions of tuberculin in order to determine the degree of hypersensitiveness of the patient's skin. Usually four simultaneous injections are given, using dilutions of old tuberculin of 1 to 10,000,000, 1 to 1,000,000, 1 to 100,000, and 1 to 10,000, representing respectively, 0.0000001, 0.000001, 0.00001 and 0.0001 gram of tuberculin. Separate sterile syringes should be used for each dilution and 0.1 c.c. of sterile salt solution should be similarly injected as a control. For routine exclusion purposes a dose of 0.00001 gram would be reliable in the majority of instances. (See Methods of Diluting Tuberculins.) The reaction when positive appears in six to eight hours, reaches its maximum in twenty-four to forty-eight hours and generally subsides in six to ten days, and consists of infiltration, hyperemia, and, in severe reactions, vesiculation. The width of the area of infiltration and the degree of inflammation are noted. It not infrequently happens that a person fails to give a positive reaction at the first test yet shows the typical local manifestations when the test is repeated. This has given rise to the impression that an injection of tuberculin sensitizes the individual. Without further discussion it may be stated that, as far as we know, there is no skin sensitiveness without infection. The appearance of a positive reaction at the second injection may be looked upon as a true reaction, and it is likely that the first injection while eliciting no response in the skin has served to stimulate the latent hypersensitiveness of the cells.

**The Subcutaneous-local Test or "Stich Reaktion."**—The test is carried out as in the intracutaneous test, with the difference that the needle is inserted into the subcutaneous tissue with the point of the needle directed toward the surface. The intracutaneous test is preferable.

**The Cutaneous Test of von Pirquet.**—This is performed as follows: The inner side of the forearm is cleansed with alcohol and ether and two small similar scarifications or scratches are made about three inches apart. Oozing of blood is to be avoided. On one spot or scratch a drop of tuberculin is placed and allowed to dry on the scarification. The tuberculin may be diluted to 25 per cent. if desired. The other spot is kept as a control. Both spots should be examined at the end of twelve, twenty-four, and thirty-six hours. A positive reaction appears after three to twenty-four hours and is usually at its height at thirty-six to forty-eight hours, and consists then of a slightly raised reddening of the skin somewhat circular in outline and usually about 10 mm. in diameter. Reactions under 5 mm. in diameter should be regarded as doubtful.

**The Subcutaneous Test.**—The object of this test was to elicit a constitutional reaction by the injection of old tuberculin under the skin. Owing to the severe reactions frequently obtained which resulted in harm to the patient it is now considered the better practice to abandon this test in favor of the Mantoux or von Pirquet methods.

**The Percutaneous Test of Moro.**—An ointment is made of equal parts of lanolin and tuberculin ("O. T."), and a small amount of it is rubbed into the skin on the chest. A positive reaction is shown by the development of reddening and papules.

**Ophthalmic Test of Calmette.**—Owing to the occurrence of serious accidents this test is little used. A drop of a 2 per cent. solution of tuberculin is applied to the lower conjunctival sac. The reaction is indicated by secretion and reddening of the inner canthus caruncle or lower lid, which may include the entire conjunctiva, with edema of the lids.

**Deductions and Limitations of the Tuberculin Test for Diagnosis.**—A positive reaction indicates the presence of a tuberculous focus but not necessarily of tuberculous disease. It tells nothing of the location, extent or activity of the lesion. Krause gives the following basis for interpreting the reaction: There is no cutaneous hypersensitiveness without a focus (tubercle); this hypersensitiveness appears coincident with the establishment of the focus; it diminishes with the healing of the focus; it varies directly with the intensity of the disease. It should be mentioned, further, that many advanced cases, particularly those in cachexia, fail to show any appreciable response to tuberculin tests, and also that measles diminishes hypersensitiveness.

**Tuberculin: Kinds and Preparation.**—There exist a large number of tuberculin preparations. The following, however, hold the highest favor and suffice for the needs of the diagnostician or practitioner:

**Tuberculin, Koch's "Old" (O. T.).**—Cultures of tubercle bacilli after six weeks' growth on 5 per cent. glycerin broth[1] are heated in the Arnold sterilizer to kill the bacilli, and filtered. This bacillus-free filtrate is evaporated to one-tenth its original bulk and after filtering through paper to remove the sediment it is ready for use. The tuberculin is therefore a heated 50 per cent. glycerin solution of the products of the bacilli in the culture fluid and such portions of the bacilli as go into solution. It is used chiefly for diagnostic purposes.

**Tuberculin B. F. (Bouillon Filtrate of Denys)** is made from a broth culture as above. The culture is not heated, but filtered first through paper and then through a Berkefeld filter to insure sterility. It differs from the original tuberculin in that it is neither heated nor concentrated, and contains only such constituents of the bacillus as are soluble or are developed in the culture medium during cultivation.

**Tuberculin B. E. (Bacillus Emulsion)** is produced by grinding up dried tubercle bacilli until no intact bacilli can be found on microscopic examination. This powder is then suspended in glycerin-water and heated to 60° C. for an hour or more to kill any viable tubercle bacilli. The proportion of bacillus to water is such that 1 c.c. of fluid contains 5 mg. of bacillus substance. This preparation contains all the constituents of the tubercle bacillus in an unchanged state, and therefore corresponds to a bacterial vaccine.

The undiluted products keep for a long time if kept cool and protected from the light. The low dilutions keep for at least one month, but the high dilutions should not be used after two weeks.

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[1] Broth should be made 1.5 per cent. acid to phenolphthalein.

**Method of Diluting Tuberculin.**—The following describes a method of dilution in terms of volume of finished tuberculin, and gives the weight equivalent. Although in the case of bouillon filtrate many use dilutions, taking into consideration the fact that the bouillon filtrate is not concentrated, or in the case of bacillus emulsion dilute according to the weight of solid substance contained, a uniform method for each seems advisable for reasons of simplicity.

If we consider the finished product in terms of cubic centimeters or grams regardless of the contents, the following is the method of dilution. Dilutions should be made with sterile saline to which 0.25 per cent. carbolic has been added.

| Dilutions. | Amount of tuberculin. | Amount of diluent. | Content of tuberculin terms of finished product. | Using bacillus emulsion content of solid substance in each dilution will be: |
|---|---|---|---|---|
| A | 1 c.c. | 9 c.c. | 1 c.c. = 0.1 c.c. or gm., or 100 c.mm. or mg. | 1 c.c. = 0.5 mgm., or 0.5 mgm. |
| B | 1 c.c. of dilution A | 9 c.c. | 1 c.c. = 0.01 c.c. or gm., or 10 c.mm. or mg. | 1 c.c. = 0.05 mgm., or 0.05 mgm. |
| C | 1 c.c. of dilution B | 9 c.c. | 1 c.c. = 0.001 c.c. or gm., or 1 c.mm. or mg. | 1 c.c. = 0.005 mgm., or 0.005 mgm. |
| D | 1 c.c. of dilution C | 9 c.c. | 1 c.c. = 0.0001 c.c. or gm., or 0.1 c.mm. or mgm. | 1 c.c. = 0.0005 mgm., or 0.0005 mgm. |
| E | 1 c.c. of dilution D | 9 c.c. | 1 c.c. = 0.00001 c.c. or gm., or 0.01 c.mm. or mgm. | 1 c.c. = 0.00005 mgm., or 0.00005 mgm. |
| F | 1 c.c. of dilution E | 9 c.c. | 1 c.c. = 0.000001 c.c. or gm., or 0.001 c.mm. or mgm. | 1 c.c. = 0.000005 mgm., or 0.000005 mgm. |

**Tuberculin Treatment.**—Tuberculin is not a cure for tuberculosis. It promotes healing and relapses are less frequent after its use. It should be used as an addition not as a substitute for the recognized methods of treatment. It is a two-edged weapon and should be employed only by those who have a thorough understanding of its possibilities for good, and unfortunately, for harm.[1]

The principle of the method is to start with the injection of minute doses one-tenth to one one-thousandth of a milligram and gradually increase the dose by 5 to 10 per cent. additions till the limits of tolerance are reached. The increase is so gradual that the course of treatment takes a long time and there is little use in applying it unless it can be continued for several months. Every effort is made to avoid reactions as evidenced by temperature rise, increased pulse-rate or other symptoms. Intolerance may also be shown by indefinite symptoms such as fatigue, loss of appetite, headache and malaise. The dosage is decreased or one or more injections omitted if symptoms of reaction appear. The treatment is without value in advanced tuberculosis. The results indicated are favorable in some cases, but the treatment is not a cure and should be employed as an addition to the other recognized methods of treatment.

**United States Government Directions for Inspecting Herds for Tuberculosis.**—"Inspection should be carried on while the herd is stabled. If it is necessary to stable animals under unusual conditions or among surroundings that make them uneasy and excited, the tuberculin test should be postponed until the cattle have become accustomed to the conditions they are subjected to, and then begin with a careful physical examination of each animal. This is essential, because in some severe cases of tuberculosis, on account of saturation with toxins, no reaction follows the injection of tuberculin, but experience has shown that these cases can be discovered by physical examination. This should include a careful examination of the udder and of the superficial lymphatic glands, and auscultation of the lungs.

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[1] For details of tuberculin treatment see Haman and Wollman, "Tuberculin in Diagnosis and Treatment," Appleton, New York, 1912.

"Each animal should be numbered or described in such a way that it can be recognized without difficulty. It is well to number the stalls with chalk and transfer these numbers to the temperature sheet, so that the temperature of each animal can be recorded in its appropriate place without danger of confusion. The following procedure has been used extensively and has given excellent results:

"(*a*) Take the temperature of each animal to be tested at least twice, at intervals of three hours, before tuberculin is injected.

"(*b*) Inject in the evening, preferably between the hours of six and nine, 0.4 c.c. of Koch's tuberculin, previously diluted to 5 c.c. with sterile water. The injection should be made with a carefully sterilized hypodermic syringe. The most convenient point for injection is back of the left scapula. Prior to the injection the skin should be washed carefully with 5 per cent. solution of carbolic acid or other antiseptic.

"(*c*) The temperature should be taken nine hours after the injection, and temperature measurements repeated at regular intervals of two or three hours until the sixteenth (eighteenth)[1] hour after the injection.

"(*d*) When there is no elevation of temperature at this time the examination may be discontinued; but if the temperature shows an upward tendency, measurements must be continued until a distinct reaction is recognized or until the temperature begins to fall.

"(*e*) If a cow is in a febrile condition tuberculin should not be used, because it would be impossible to determine whether, if a rise of temperature occurred, it was due to the tuberculin or to some transitory illness.

"(*f*) Cows should not be tested within a few days before or after calving, for experience has shown that the results at these times may be misleading.

"(*g*) In old, emaciated animals and in retests, use twice the usual dose of tuberculin, for these animals are less sensitive.

"(*h*) Condemned cattle must be removed from the herd and kept away from those that are healthy.

"(*i*) In making postmortems the carcasses should be thoroughly inspected, and all the organs should be examined."

**Antituberculous Serum.**—Every conceivable way of obtaining the true products of the tubercle bacilli has been tried, so as to cause the injected animals to produce antibodies both antitoxic and bactericidal. In spite of much conflicting testimony, it is probably safe to assert that no serums now obtainable have any great value.

**Prophylaxis.**—All energies should be directed to the prevention of tuberculosis, not only by the enforcement of proper sanitary regulation as regards the care of sputum, milk, meat, disinfection, etc., but also by continued experimental work and by the establishment of free consumptive hospitals, and by efforts to improve the character of the food, dwellings, and conditions of the people in general, we should endeavor to build up the individual resistance to the disease. It may be years before the public are sufficiently educated to coöperate with the sanitary authorities in adopting the necessary hygienic measures to stamp out tuberculosis entirely; but, judging from the results which have already been obtained in reducing the mortality from this dread disease, we have reason to believe that in time it can be completely controlled.

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[1] The directions allow temperatures to be stopped the sixteenth hour, but even when there is no reaction at all it is much safer to always take temperatures for eighteen hours. We have found now and then a tuberculous cow that reacted on the eighteenth hour for the first time.

Among the numerous medical agents that have been tried without avail to protect animals against the action of the tubercle bacillus may be mentioned tannin, menthol, sulphuretted hydrogen, mercuric chloride, creosote, creolin, phenol, arsenic, eucalyptol, etc.

**Agglutination and Complement-fixation Reaction for Diagnosis.**—The results obtained with the agglutination reaction by various observers have been very conflicting. At present the test cannot be advised as useful in diagnosis, as the sera of cases suffering from tuberculosis frequently fail to give a reaction, while the sera from those having no detectable tuberculosis frequently cause a good reaction. A reaction in dilutions of 1 to 10 or 1 to 15 is considered a positive test. (For Complement fixation see Chapter XIV.

**The Tubercle Bacillus of Domestic Animals and its Relation to Human Tuberculosis.**—Among the domestic animals tuberculosis is most common in cattle. On account of the milk which they provide for our use, and which is likely to contain bacilli, the relation of these to human tuberculosis is a matter of extreme importance.

The chief seat of the lesions is apt to be the lymphatic nodes or lungs, and with them the pleura; less often the abdominal organs and the udder are affected. In pigs the abdominal organs are more often involved, then the lungs and lymphatic glands. In sheep, horses and goats tuberculosis is rare.

**Differences between Tubercle Bacilli of Human and Bovine Type.**—As has been already noted in the tables given of the incidence of bovine and human infection, it is possible to tell in any case the type of infection. The essential differences are in cultural characteristics and in virulence for rabbits and calves.

**Cultural Differences.**—The bovine bacillus grows very poorly when isolated, the human bacillus very freely. This is noted on plain egg, but to a less extent than on glycerin egg. The glycerin restrains or adds little to the growth of bovine bacilli, but increases markedly the amount of growth of the human bacillus. In fact, primary cultures on glycerin egg of bovine material commonly fail to grow. This difference is very noticeable in the first few generations and is sufficient in the great majority of instances for differentiation to one who has had some experience with such cultures. Further, the majority of human strains can be transplanted to glycerin potato or glycerin broth and give vigorous growth in the first few generations, whereas the bovine bacillus fails to grow or growth is very slight. After further cultivation the bovine bacillus gradually increases its amount of growth until it is indistinguishable from the human type. This increase in luxuriance may be rapid or very slow.

**Rabbit Virulence.**—The bovine bacillus is exceedingly virulent for rabbits by any method of inoculation; the human bacillus only slightly so. The best method of differentiation is by intravenous inoculation.

A small amount of culture is weighed after the moisture has been extracted with filter paper, and a suspension made in normal saline and diluted so that 1 c.c. = 0.01 mg. of culture; this amount is then injected into the ear vein of a rabbit. If the rabbit survives for from forty to fifty days, and on autopsy shows only lesions in the lungs or kidneys or both, the strain is of the human type. With the bovine type of bacillus the rabbit will die in the majority of instances before or about this time, if not it may be killed. On autopsy a progressive generalized tuberculosis will be found. The lesions in the lungs will be very marked, the tubercles having become confluent with caseous centers. The liver or spleen or both will be peppered with tubercles. Tubercles will be present in the great majority of cases in the superficial lymph nodes and also in those of abdomen and thorax. There may be tubercles on the heart, in the rib marrow, or over the peritoneum.

These two differences alone are sufficient to differentiate in every case the type of bacillus. It must be insisted upon again that the cultural characteristics be observed in the early generation and, further, that the virulence be tested in early generations. In case the bovine culture does not afford sufficient material for weighing, a suspension can be made and compared with a weighed suspension.

**Virulence for Calves.**—In proving the non-identity of the two bacilli, calf experiments were resorted to. This was necessary as the supposed bovine cultures from children would have to be virulent for calves to the same extent as cultures from bovine material. The commonly used method was the subcutaneous inoculation in the side of the neck with 50 mg. of culture. The human type of bacillus caused only a local lesion or at most a spreading to the nearest lymph node. The bovine bacillus, on the other hand, caused a generalized tuberculosis which was or was not fatal. Sufficient data has been accumulated to make this test practically unnecessary for the determination of type.

**Differences in Morphology.**—The bovine bacillus tends to be shorter, thicker, and solidly stained; the human type tends to be longer, slimmer, usually bent, and shows beading and irregularities in staining. We have found this difference most marked on glycerin egg, slight or imperceptible on other media.

Besides the above differences Theobald Smith made the interesting discovery that the production of acid differed with the two types when grown on glycerin broth. The bovine type renders the bouillon less and less acid; this may even progress until the medium becomes slightly alkaline to phenolphthalein. The human type causes a preliminary fall in the acidity; as growth progresses the acidity is then gradually increased, and may exceed the original acidity of the broth used. This difference is evident in tuberculin made from the two types of bacilli. The bovine tuberculin is alkaline or very slightly acid while human tuberculin is markedly acid. The change is only noticed when glycerin is used in the media. The work of more recent investigators would seem to show that this difference, like all differences between the types, is purely quantitative, and that different strains vary in their reactions and give intermediate reactions between these two extremes.

**Bird (Avian) Tuberculosis.**—Tuberculosis is very common among fowl. The bacillus grows easily and freely on glycerin media. It tends to form a moist or even slimy growth, and commonly produces an orange pigment. It is able to grow at a higher temperature than mammalian tubercle bacilli, the latter failing to grow above 41° C., the former growing at even higher temperatures. Guinea-pigs are less susceptible to inoculation with avian tubercle bacilli, and the virulence for these animals is usually quickly lost. Rabbits are much more susceptible. Rats and mice are spontaneously infected with avain tubercle bacilli and are supposed to be an important factor in spreading the disease. Birds are refractory, with few exceptions, to infection with the mammalian tubercle bacillus. Parrots, however, are susceptible to infection with all three types and commonly have spontaneous tuberculosis caused by the human type of bacillus.

**Stability of the Different Types of Bacilli.**—The fact that the agglutination reactions and the tuberculin reactions of the different types is similar shows their close relationship. This has led to the endeavor to change one type into the other. This is usually attempted by passage through animals. The results have been peculiar. Some cultures have been passed through a series of calves without any change except for a moderate increase in virulence. Other cultures seem to have completely changed their type. We believe that this is not a change of type, but an additional bovine infection. Strong negative evidence is the fact that the bovine bacillus when infecting man loses none of its characteristics, though present in the human body for years.

**Tuberculosis of Cold-blooded Animals.**—The bacilli of this group are of interest mainly because of the claims of Friedmann that immunity against the tubercle bacillus is produced by their injection, and the publicity given to his claims that vaccines of these organisms have a curative influence in tuberculosis. The results of the use of the so-called Friedmann vaccine have, however, been unsatisfactory. A similar attempt to employ the cold-blooded types for immunization is the use in cattle as advised by Klimmer.

These types have been isolated from spontaneous, tuberculous-like lesions of frogs, lizards, turtles, fishes, etc. They have little resemblance to the mammalian types of tubercle bacilli other than their acid-fastness. They grow rapidly and luxuriantly on ordinary media and their optimum of growth is at 20° to 30° C., higher temperatures inhibiting their growth. They are not pathogenic for warm-blooded animals, although toxic symptoms or limited lesions may be produced by the injection of large doses as with most other non-pathogenic acid-fasts. Tuberculins prepared from them are not toxic for tuberculous mammalia except in large doses, so that a specific reaction can be excluded. It is possible but very improbable that they might stimulate the production of some antibodies which would protect against mammalian bacilli to a limited extent.

**Methods of Examination for Tubercle Bacilli.**—One of the most important results of the discovery of the tubercle bacillus relates to the practical diagnosis of tuberculosis. The staining peculiarities of this bacillus render it possible by the bacteriological examination of microscopic preparations to make an almost positive diagnosis in the majority of cases. A still more certain test in doubtful cases is the subcutaneous or intraperitoneal injection of guinea-pigs, which permits of the determination of the presence of numbers of bacilli, so small as to escape detection by microscopic examination. For the animal test, however, time is required—at least three weeks, and, if the bacilli present are very few in number, at least six weeks—before any positive conclusion can be reached, for when only a few bacilli are present tuberculosis develops slowly in animals. In disinfection experiments where many dead bacilli are injected, care must be taken to exclude the local effect of dead bacilli. In doubtful cases a second guinea-pig should be injected with material from the first.

**Microscopic Examination of Sputum for the Presence of Tubercle Bacilli.**—1. **Collection of Material.**—The sputum should be collected in a clean bottle (two-ounce) with a wide mouth and a water-tight stopper, and the bottle labelled with the name of the patient or with some other distinguishing mark. The expectoration discharged in the morning is to be preferred, especially in recent cases, and the material should be coughed up from the lungs. Care should be taken that the contents of the stomach, nasopharyngeal mucus, etc., are not discharged during the act of expectoration and collected instead of pulmonary sputum. If the expectoration be scanty the entire amount discharged in twenty-four hours should be collected. In pulmonary tuberculosis the purulent, cheesy, and mucopurulent sputum usually contains bacilli; while pure mucus, blood, and saliva, as a rule, do not. When hemorrhage has occurred, if possible some purulent, cheesy, or mucopurulent sputum should be collected for examination. The sputum should not be kept any longer than necessary before examination, for, though a slight delay or even until putrefaction begins, does not vitiate the results so far as the examination for tubercle bacilli is concerned, it almost destroys any proper investigation of the mixed infection present; it is best, therefore, to examine it in as fresh a condition as possible, and it should be kept on ice until examined if cultures are to be made.

2. **Methods of Examination.**—*Examination for Tubercle Bacilli.*—Pour the specimen into a clean, shallow vessel, having a blackened bottom—a Petri dish placed upon a sheet of dull black paper answers the purpose—and select from the sputum some of the true expectoration, containing, if possible, one of the small white or yellowish-white cheesy-looking masses or "balls." From this make rather thick cover-glass or slide "smears" in the usual way. In doubtful cases a number of these coarse or fine particles should be placed on the slide. The material being thick should be evenly spread and very thoroughly dried in the air before heating. (For methods of staining see above and p. 78.)

Occasionally one is able to demonstrate the presence of tubercle bacilli with the Hermann stain where Ziehl's carbol-fuchsin gives negative results. At least two smears should be made and examined, if possible, before a negative report is given. Many of the incipient cases will require several examinations before bacilli are found. Some will remain consistently negative. It must be remembered that lesions may exist and that without ulceration the bacilli do not find their way into the sputum.

*Methods for Concentrating the Bacilli.*—Uhlenhuth advises the use of *antiformin*. This is a patented preparation consisting of a mixture of sodium hydroxide and sodium hypochlorite solution. If this is mixed with sputum so that the total strength is about 15 per cent. of antiformin, the sputum quickly becomes fluid. This should be thinned with water or alcohol to help reduce the specific gravity of the mixture and centrifuged. The sediment is then mixed with water and recentrifuged, and the washed sediment used for smears. Besides the dissolving action, antiformin kills most of the bacteria in the sputum, but not the tubercle bacilli, though they are slowly affected, so that sediment may be used for cultural purposes or injection into guinea-pigs.

A comparison of the above methods made by us gave the following results: Of twenty-eight sputa negative with carbol-fuchsin, two showed bacilli after a few minutes' search with the crystal violet stain. On restaining with carbol-fuchsin and giving only a light counter-stain with methylene blue the negative slides were also positive. Of the remaining twenty-six, four (15 per cent.) were quickly positive in the antiformin sediment when stained with crystal violet, whereas only three were positive with carbol-fuchsin and only after restaining as above. It is advisable, therefore, in using carbol-fuchsin to have only a light counter-stain to make the method most efficient, and control the results with crystal violet if negative.

In place of sedimenting the bacilli, the dissolved sputum may be shaken up with a hydrocarbon. When the hydrocarbon separates out from the sputum the waxy tubercle bacilli adhere to it and are collected in a layer between the dissolved sputum and the hydrocarbon. If the hydrocarbon is heavy, chloroform, they are carried down, if light, ligroin, they are carried up.

Kinyoun has modified the original ligroin method as given below. We have had very satisfactory results with its use. As a routine method it saves time and gives a high percentage of positive results.

Bottles of about 15 c.c. capacity containing about 2 c.c. of a 1 per cent. solution of cresol are used for collection. The cresol is added to limit decomposition of the sputum if its transit to the laboratory is delayed. When received 1 c.c. of ligroin (specific gravity not less than 0.715 or more than 0.72) is added and the bottle filled with an alkaline solution of hypochlorite of lime. If the bottle is full, about one-third must be poured out to allow for the addition of the solution. The hypochlorite solution is prepared as follows: Three packages of chlorinated lime are weighed, and for each 90 grams, 65 grams of sodium carbonate are taken. The lime is mixed with 500 to 600 c.c. of water and the carbonate is dissolved in 1500 c.c. of water by boiling. The carbonate is then added to the lime and thoroughly mixed. After standing twelve to twenty-four hours the solution is filtered off. The amount of available chlorine is estimated and the solution diluted so that the chlorine is 0.56 per cent. Then

7.5 grams of caustic soda are added to each 100 c.c. of the filtrate. The solution should be kept cool and in the dark. Fresh lots should be prepared about every three months.

After the addition of the ligroin and the solution of chlorine the bottles of sputum are placed in a shaking machine and thoroughly shaken for five to ten minutes. The bottles can then be allowed to stand until the ligroin rises, which takes several hours, or this can be hastened, placing the bottles in a centrifuge, with special cups to accommodate the bottles, and run for about ten minutes at moderate speed.

When the ligroin rises to the top a soapy layer develops at the point of contact with the fluidified sputum and the tubercle bacilli are collected in this layer. The soapy layer is taken up with a platinum loop and smears made on glass slides fixed by heat and stained. Individual slides must be used and the slides must be stained separately or error will result, as the bacilli are not firmly fixed to the slides.

All the antiformin methods must be used with caution, as it is easy to see how error can creep in from contamination with other acid-fast bacilli. (See also the Petroff method given above.)

**Detection of Tubercle Bacilli in Urine and Feces, etc.**—The catheterized urine is centrifuged. If little sediment appears, the upper portion of the fluid is removed and more urine added and again centrifuged. If the urine is rich in salts of uric acid, the same may be diminished by carefully warming the urine before treating it. If too alkaline add a little acetic acid. A possible source of error is the presence of smegma bacilli.

The feces are examined for any purulent or mucous particles. If none are found, larger masses of feces are removed and then the rest diluted and centrifugalized. The antiformin methods are a great aid in the examination of feces. The examiner must remember that bacilli swallowed with the sputum may appear in the feces.

In examining cerebrospinal fluid for tubercle bacilli it must be remembered that the majority of the bacilli are entangled in the delicate clot that forms. Whenever possible the fluid after withdrawal should be allowed to stand until this filmy clot develops, which is then fished out and examined. If this is impossible the fluid should be centrifuged and the sediment stained. This is also the case in other serous fluids, but in ascitic or pleuritic fluid they are usually very few in number. (For sputum washing see p. 152.)

**Inoculation of Animals.**—The inoculation of suspected material into guinea-pigs produces tuberculosis; even if the number of bacilli is very small. When no bacilli can be detected by microscopic examination this can be done for diagnostic purposes. The material should be injected subcutaneously as already described.

**Cultivation.**—This requires so much time that it is not generally used except in important investigations upon the nature of the tubercle bacilli. The special methods have already been given.

# CHAPTER XXVIII.

# OTHER ACID-FAST BACILLI: BACILLUS OF LEPROSY, BACILLUS OF RAT LEPROSY, BACILLUS OF JOHNE'S DISEASE IN CATTLE, AND THE GROUP OF NON-PATHOGENIC ACID-FAST BACILLI.

## LEPROSY BACILLUS—B. LEPRÆ.

THE bacillus of leprosy was discovered by Hansen and Neisser (1879) in the leprous tubercles of persons afflicted with the disease. This discovery was confirmed by many subsequent observers.

**Morphology (in Tissues).**—Small, slender rods resembling the tubercle bacilli in form, but somewhat shorter and not so frequently curved. The rods have pointed ends, and in stained preparations unstained spaces, similar to those observed in the tubercle bacillus, are seen. They *stain* readily with the anilin colors and also by Gram's method. Although differing slightly from the tubercle bacillus in the ease with which they take up the ordinary anilin dyes, they behave like tubercle bacilli in retaining their color when subsequently treated with strong solutions of the mineral acids and alcohol. The difference in staining characteristics is too slight to be relied upon for diagnostic purposes (see Plate VI).

**Bacilli Isolated from Leprous Lesions.**—No acid-fast organism was grown from leprous lesions until Clegg reported, in 1908, that he had been able to cultivate an acid-fast bacillus by growing it in symbiosis with ameba and cholera. Since then Duval, Kedrowski, Twort, and many others have reported the finding of various more or less acid-fast organisms in leprous lesions. These organisms may be grouped as follows:[1]

**Bacilli of the Diphtheroid Type.**—In serum media the colonies are yellowish white and develop best at 37° C., although a slight growth occurs at room temperature. When growth occurs on broth, the medium remains clear, and a pellicle is produced. The morphology is variable. They are either solidly stained or irregularly stained like other types of diphtheroids. They are Gram-positive and may show some resistance to decolorization after staining with carbol-fuchsin, especially the metachromatic granules.

*Pathogenicity,* none or questionable.

**Acid-fast Chromogenic Bacilli.**—This type of bacillus is difficult to isolate but after isolation grows freely at both 37° and 20° C. on most of the ordinary media. The growth is luxuriant, moist, and a yellow to deep orange color develops. The individual bacilli vary in morphology from coccoid to filamentous bacilli, some showing metachromatic granules, others showing clear areas. They are acid-fast but less so than the tubercle bacillus.

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[1] The results of all the investigations cannot be given. For a fuller discussion and bibliography see the excellent *résumé* of Wollbach and Honeij: Jour. Med. Research, 1914, xxix, 367.

*Pathogenicity.*—Lesions similar to leprosy produced in Japanese dancing mice and in monkeys.

**Anaërobic Bacilli.**—In this place it is sufficient to state that such organisms have been isolated.

**Acid-fast Non-chromogenic Bacilli.**—These types are characterized by their feeble, slow growth on artificial media, and growth only takes place at 37° C., and then only on special media.

Morphologically they vary from plump to long, slender bacilli, often beaded or bipolar in appearance.

*Pathogenicity,* none.

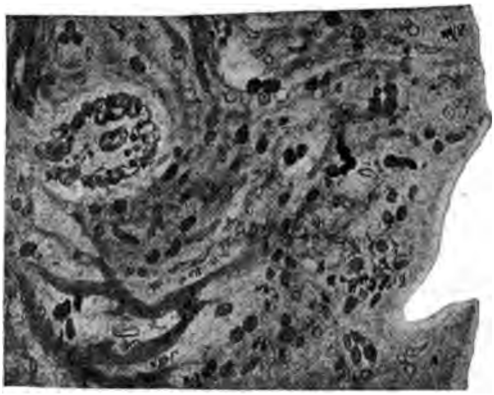

FIG. 146.—Leprosy bacilli in nodule. (Kolle and Wassermann.)

What conclusion is to be drawn from such variable results is difficult to say. Has the diphtheroid bacillus any relationship to the more acid-fast types? This is a possibility when one considers that under certain circumstances it shows some resistance to decolorization. Then, too, the leprosy bacilli in tissue may decolorize easily, although they are abundant, as shown by staining with polychrome methylene blue. On the other hand, the repeated isolation of diphtheroids from the lymph nodes in other conditions raises a strong element of doubt as to the etiological significance of this organism. A careful comparison of the diphtheroids from various conditions, especially their resistance to decolorization, might give us some help.

As to the chromogenic acid-fasts, the character of their growth, viz., the luxuriant growth upon ordinary media at low temperatures is not that of a highly specialized parasite. As to the apparently successful animal inoculations it must be remembered that lesions have been produced by acid-fast bacilli, known to be non-pathogenic. Why such bacilli should be frequently isolated from leprous lesions is still to be explained.

The non-pigmented types are more consistent with our idea of what the bacillus should be but whether they are actually the etiological organism remains to be seen.

The serum reactions, such as agglutination and complement fixation, have added no evidence as to the etiological significance of any one of the bacilli isolated. Each of the bacilli mentioned has been agglutinated by sera of lepers. The complement-fixation reactions await a successful specific test for the individual acid-fast organisms.

**Pathogenesis.**—Numerous inoculation experiments have been made on animals with portions of leprous tubercles, but there is no conclusive evidence that leprosy can be transmitted to the lower animals by inoculation. The inference that this bacillus bears an etiological relation to the disease with which it is associated is based chiefly upon the demonstration of its constant presence in leprous tissues.

The bacilli are found in all the diseased parts, and usually in large numbers, especially in tubercles on the skin, in the conjunctiva and cornea, the mucous membranes of the mouth, gums, and larynx, and in the interstitial processes of the nerves, testicles, spleen, liver, and kidneys. The rods lie almost exclusively within the peculiar round or oval cells of the granulation tissues which compose the leprous tubercles, either irregularly scattered or arranged parallel to one another. In old centers of infection the leprosy cells containing the bacilli are larger and often polynuclear. Giant cells, such as are found in tuberculosis, are claimed to have been observed by a few investigators (Boinet and Borrel). In the interior of the skin tubercles, the hair follicles, sebaceous and sweat glands are often attacked, and bacilli have sometimes been found in these (Unna, etc.). Quite young eruptions often show a few bacilli. A true caseation of the tubercles does not occur, but ulceration results. During acute exacerbations with development of new lesions bacilli have been observed in the blood.

In the anesthetic forms of leprosy the bacilli are found most commonly in the nerves and less frequently in the skin. They have been demonstrated in the sympathetic nervous system, in the spinal cord, and in the brain. The Bacillus lepræ occurs also in the blood, partly free and partly within the leukocytes, especially during the febrile stage which precedes the breaking out of fresh tubercles (Walters and Doutrelepont). The bacilli have also been found in the intestines, in the lungs, and in the sputum, but not in the urine.

With regard to the question of the direct inheritance of the disease from the mother to the unborn child there is considerable difference of opinion. Some cases have been reported, however, in which a direct transmission of the bacillus during intra-uterine life seems to be the only or most plausible explanation of the infection. At the same time, we have no positive experimental evidence to prove that such an infection does take place. Although many attempts have been made to infect healthy individuals with material containing the bacilli of leprosy, the results are not conclusive. Even the experiments made by Arning, who successfully infected a condemned criminal in the Sandwich Islands with fresh leprous tubercles, and which have been regarded as positive evidence of the transmissibility of the disease in this way, are by no means conclusive; for, according to Swift, the man had other opportunities for becoming infected. The negative results, together with the fact that infection does not more frequently occur in persons exposed to the disease, may possibly be explained by the assumption that the bacilli contained in the tuberculous tissues are mostly dead, or much more probably that an individual susceptibility to the disease is requisite for its production.

The widespread idea, before the discovery of the leprosy bacillus, that the disease was associated with the constant eating of dried fish or a certain kind of food, has now been entirely abandoned.

The relation of leprosy to tuberculosis is sufficiently evident from their great similarity in many respects. This is rendered still more remarkable by the fact that leprosy reacts, both locally and generally, to an injection of tuberculin in the same manner as tuberculosis, but to a somewhat less extent.

**Rat Leprosy.**—The interest in this disease lies in the fact that diphtheroid and chromogenic acid-fast bacilli similar to those described above have been isolated from leprous rats.

**Bacillus of Johne's Disease, Chronic Enteritis or Paratubercular Dysentery of Cattle.**—This disease is comparatively common in this country and is characterized by chronic diarrhea and emaciation, commonly leading to death. The intestinal mucosa is thickened, and the lesions are not limited. Tubercle formation and necrosis are absent. The bacilli are present in the lesions in enormous numbers. Twort succeeded in cultivating the organism and his work was verified by Holth and Meyer. A tuberculin made from this organism will probably be of diagnostic value. Animals having this disease do not react to the ordinary tuberculin test but do react to large doses of tuberculin made from the avian type of tubercle bacillus. The bacillus is not pathogenic for guinea-pigs or rabbits, although local abscesses may be produced.

**Non-pathogenic Acid-fast Bacilli.**—These have no importance further than historical interest and the fact that they may be present in materials suspected of containing tubercle bacilli and thus lead to error. They vary widely in their acid-fastness, especially when artificially cultivated. Differential staining methods have been devised to separate them from the tubercle bacillus and although in a general way the decolorization by prolonged action of acid and alcohol is presumptive evidence against suspected bacilli being tubercle bacilli, it is an unsafe procedure. Tubercle bacilli vary in their acid-fastness but the non-pathogenic types vary even more widely, some being extremely resistant to decolorization. Many of the non-pathogenic types grow rapidly at low temperatures and in cultures can thus be quickly differentiated from tubercle bacilli.

They can be separated from tubercle bacilli by inoculating animals in which no progressive lesions will develop, although limited lesions may be produced if they are injected in large numbers. The guinea-pig may be injected with 2 c.c. of tuberculin and if infected with tuberculosis will die, but if by other acid-fast bacilli, will show little or no reaction. If a second group of guinea-pigs are inoculated with a small amount of the infected tissue from the inoculated pigs there will develop progressive tuberculosis if the doubtful bacilli were tubercle bacilli, and practically no lesions if they were grass bacilli. Cultures from the lesions may also be an aid in differentiation.

**Bacillus of Lustgarten.**—This bacillus was found by Lustgarten in 1884 in syphilitic lesions or ulcers. It is undoubtedly a saprophyte. It is very similar morphologically to the smegma bacillus and may be identical with it. It is of historical interest only.

**Smegma Bacillus.**—This bacillus is present in smegma from the prepuce or vulva. Its only interest is the danger of mistaking it for tubercle bacilli in the examination of urine, especially if the latter be carelessly collected.

**Timothy and Other Grass Bacilli.**—On various grasses, in cow manure, in butter and in milk there have been found bacilli with varying degrees of acid-fastness. Similar bacilli have also been demonstrated in water. They make the direct microscopic examination of such material for tubercle bacilli of little value and the nature of any acid-fast organisms so found must be determined by animal inoculations.

# CHAPTER XXIX.

## GLANDERS BACILLUS (BACILLUS MALLEI). B. ABORTUS (BANG).

### GLANDERS BACILLUS.

THE Bacillus mallei was discovered and proved to be the cause of glanders by several bacteriologists at almost the same time (1882). Bouchard, Capitan and Charin obtained it in mixed cultures, while it was first accurately studied in pure culture by Löffler and Schütz. It is present in recently formed nodules in animals affected with glanders, in the nasal discharge, in pus from the specific ulcers, etc., and occasionally in the blood.

**Morphology.**—Small bacilli with rounded or pointed ends, from nutrient agar cultures, 0.25μ to 0.5μ broad and from 1.5μ to 5μ long; usually single, but sometimes united in pairs, or growing out to long filaments, especially in potato cultures. The bacilli frequently break up into short almost coccus-like elements (Fig. 147).

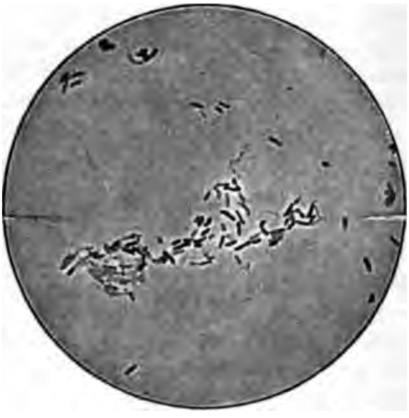

FIG. 147. — Glanders bacilli. Agar cuture. × 1,100 diameters.

**Staining.**—The bacillus mallei *stains* with difficulty with the anilin colors, best when the aqueous solutions of these dyes are made feebly alkaline; it is decolorized by Gram's method. This bacillus presents the peculiarity of losing very quickly in decolorizing solutions the color imparted to it by the anilin staining solution. For this reason it is difficult to stain in sections. Löffler recommends his alkaline methylene-blue solution for staining sections, and for decolorizing, a mixture containing 10 c.c. of distilled water, 2 drops of strong sulphuric acid, and 1 drop of a 5 per cent. solution of oxalic acid; thin sections to be left in this acid solution for five seconds.

**Biology.**—A non-motile bacillus, whose molecular movements are so active that they have often been taken for motility. It is aërobic, but moderate multiplication occurs in the depths of culture media. Grows well on culture media at 37° C. Development takes place slowly at 22° C. and ceases at 43° C. The bacillus does not form spores.

Exposure for ten minutes to a temperature of 55° C., or for five minutes to a 3 to 5 per cent. solution of carbolic acid, or for two minutes to a 1 to 5000 solution of mercuric chloride destroys its vitality. As a rule the bacilli do not grow after having been preserved in a desiccated condition for a week or two; in distilled water they may live twenty-five days. It is doubtful whether the glanders bacillus finds conditions in nature favorable to a saprophytic existence.

A solution of chlorinated lime, containing 1 part of free chlorine per 1000, is useful as a disinfectant of stables and utensils; it kills the bacillus in from one to two minutes. Strong sodium carbonate solution (washing soda) is also useful.

**Cultivation.**—(For obtaining pure cultures see below.)—It grows well at 37° C. on *potato-glycerin-veal agar*, an acidity of 1.5 to 2.5 (phenolphthalein), about $P_h$ 6.4 being the most favorable. Upon this medium, at the end of twenty-four to forty-eight hours, whitish, transparent colonies are developed, which in six or seven days may attain a diameter of 7 or 8 mm. On *blood serum* a moist, opaque, slimy layer develops, which is of a yellow-brown tinge. The growth on cooked *potato*, that is, sterilized, is especially characteristic. At the end of twenty-four to thirty-six hours at 37° C. a moist, yellow, transparent layer develops; this later becomes deeper in color, and finally takes on a reddish-brown color, while the potato about it acquires a greenish-yellow tint. In *bouillon* the bacillus causes diffuse clouding sometimes with a pellicle, ultimately with the formation of a more or less ropy, tenacious sediment. The broth should also be as acid in reaction as the glycerin-veal agar above. The addition of potato juice to either of these media is most favorable to the growth of the organism. *Milk* is coagulated with the production of acid.

**Pathogenicity.**—The bacillus of glanders is pathogenic for a number of animals. Among those which are most susceptible are horses, asses, guinea-pigs, cats, dogs, ferrets, moles, and field mice; much less susceptible are sheep, goats, swine, rabbits, white mice, and house mice; cattle are immune. Man is susceptible and may develop either the acute or chronic form. Infection not infrequently terminates fatally, usually in about 60 per cent. of the cases. Doubtless many cases are not recognized as glanders, but are mistaken for other diseases such as pyemia, rheumatism, typhoid and syphilis. (Fitch.[1])

When pure cultures of Bacillus mallei are injected into horses or other susceptible animals true glanders is produced. The disease is characterized in the horse by a rise in temperature and by the formation upon the nasal mucous membrane of ulcers which have irregular, thickened margins and secrete a thin, virulent mucus; the submaxillary lymphatic glands become enlarged and form tumors which are often lobulated. Other lymphatic glands become inflamed, and some of them suppurate and open externally, leaving deep, open ulcers; the lungs are also involved, and the breathing becomes rapid and irregular. *Acute*

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[1] Cornell Veterinarian, July, 1914.

*generalized glanders* may cause death in one to six weeks. Young horses are especially susceptible. At *postmortem* the lungs show usually either tubercle-like nodules or pneumonic areas. Nodules may occur also in the liver, spleen and lymph glands. Of the bones, the ribs are the most often involved and contain caverns filled with a tenacious yellowish substance. In *farcy*, which is a more chronic form of the disease, circumscribed swellings, varying in size from a pea to a hazel-nut, appear on different parts of the body, especially where the skin is thinnest; these suppurate and leave angry-looking ulcers with ragged edges, from which there is an abundant purulent discharge. The lymphatics leading from these ulcers become inflamed, stand out as tense hot cords under the skin and from them new "farcy buds" may develop. Chronic cases may run on for years until an acute exacerbation due to overwork or adverse conditions brings death.

**Isolation of Pure Cultures.**—Attempts to isolate B. mallei by plating out the discharge from the nostrils or an open ulcer meet with little success since comparatively few bacilli are present usually. They are mixed besides with other bacteria which outgrow B. mallei on the plates. In such cases guinea-pig inoculations are useful if made as follows:

**Straus Reaction.**—A male guinea-pig is injected intraperitoneally with either emulsified material from a suspicious lesion or a suspension of a culture which has been obtained by plating. If B. mallei is present tumefaction of the testicles occurs usually in two to five days with evidence of pus formation. This is the "Straus Reaction." The animal is then etherized and cultures made, under aseptic conditions from the pus in the testicles. An objection to this method is that the animal may die of septicemia due to the injection of impure material, but if an uncontaminated specimen can be obtained, as from the lymphatic glands, this method is satisfactory according to most authors. Nevertheless, while a positive result is conclusive evidence of glanders, failure of the pig to develop lesions is not proof of the absence of B. mallei, for other workers find such inoculations fail in about one-half the animals injected.

Of test animals guinea-pigs and field mice are the most susceptible. In guinea-pigs *sub utaneous* injections are followed in four or five days by swelling at the point of inoculation, and a tumor with caseous contents soon develops; then ulceration of the skin takes place and a chronic purulent ulcer is formed. The essential lesion is the granulomatous tumor, characterized by the presence of numerous lymphoid and epithelioid cells, among and in which are seen the glanders bacilli. The lymphatic glands become inflamed and general symptoms of infection are developed in from two to four weeks; the glands suppurate, and in males the testicles are involved. This fact is used as an aid to diagnosis. (Straus Reaction.) Finally purulent inflammation of the joints occurs and death ensues from exhaustion. The formation of the specific ulcers upon the nasal mucous membrane, which characterizes the disease in the horse, is rarely seen when guinea-pigs are inoculated. In these the process of the disease is often prolonged or remains localized on the skin. Guinea-pigs succumb more rapidly to intraperitoneal injection, usually in from eight to ten days.

*Attenuation of virulence* occurs in cultures which have been kept for some time on artificial media and inoculation with such cultures may give a negative result, or, when considerable quantities are injected, may produce a fatal result at a later date than is usual when small amounts of a recently isolated culture are injected.

**Mode of Spread.**—Glanders occurs as a natural infection only in horses and asses. The disease is occasionally communicated to man by contact with affected animals, usually by inoculation on an abraded surface of the skin. The contagion may also be received on the mucous membrane. Infection has sometimes occurred as the result of accidents in bacteriological laboratories. It is transmissible also from man to man. Washerwomen have been infected from the clothes of a patient. The infective material exists in the secretions of the nose, in the pus of glanders nodules, and frequently in the blood; it may occasionally be found in the secretions of glands not yet affected, as in the urine, milk, and saliva, and also in the fetus of diseased animals (Bonome). From recent observations it is found that glanders is by no means an uncommon disease among apparently sound horses, sometimes taking a mild course and remaining latent for a considerable time. Therefore, horses appearing healthy, may spread the disease through the public drinking troughs and blacksmith shops.

**Immunity.**—Attempts have been made to produce artificial immunity against glanders but so far with unsatisfactory results. Various workers, as Straus, Fenger and also Ladowski, have reported the production of immunity in the smaller animals, such as dogs, cats and rabbits, by the injection of either living or killed cultures. In the horse, the most important animal from the economic standpoint, other observers have reported not only immunity but cures by the use of vaccine and also subcutaneous mallein; however, corroboration is still lacking. Such substances as mallein and vaccine when injected do produce immune bodies which can be demonstrated by the serodiagnostic tests. It is unfortunate, however, that the presence of these immune bodies in the blood does not indicate, necessarily, a practical immunity of the animal against infection. (Mohler and Eichhorn.[1])

Since the indiscriminate use of *vaccine* causes confusion in the blood tests of horses, it is not now being distributed by the Health Department of the City of New York.

In man the therapeutic value of vaccine is not yet fully determined. But few cases so far have been reported—one by Bristow and White[2] and one by Cramp,[3] recovered after the use of an autogenous glanders vaccine. The use of *mallein* in man subcutaneously, has been reported in six cases. (Robins[4]) . In one it gave a reaction and in three it was of supposed benefit therapeutically.

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[1] Bull. U. S. Dept. Agriculture, 1914, No. 70.

[2] New York State Jour. Med., 1910, p. 236.

[3] Jour. Am. Med. Assn., 1911, lvi, 1379.

[4] Studies from the Royal Victoria Hospital, Montreal, 1906, ii, 1.

**Diagnosis of Glanders.**—The chief methods (Mohler and Eichhorn[1]) are: physical examination; serodiagnostic tests—complement fixation and agglutination reaction; mallein reactions—eye, intrapalpebral and subcutaneous; "Straus Reaction" (inoculation of guinea-pigs with either suspected material or cultures); postmortem examination.

**Physical Examination.**—In horses those cases with clear-cut clinical symptoms offer little difficulty to the veterinarian. However, the easily applied eye mallein reaction should be used for confirmation. These two methods are sufficient under such circumstances.

**Serodiagnostic Tests.**—It is the latent or occult cases, showing only a little fever, or none, that require additional tests for diagnosis. Since these cases are frequently the distributors of the disease their early recognition and extermination is imperative. To detect these cases the application of the serodiagnostic reactions supplemented by the use of eye mallein is necessary.

*Collection of Blood for Serodiagnostic Tests.*—In obtaining blood from horses a large-sized hypodermic needle, which has been sterilized, is inserted into the jugular vein which has been brought into view by pressing the thumb upon it from below; the blood is allowed to flow through the needle into a sterile neutralized tube or flask, 8 to 10 c.c. being sufficient.

In the case of human beings, the median basilic vein at the bend of the elbow is used. Under aseptic conditions 5 to 10 c.c. of blood is drawn either by means of a sterile hypodermic syringe, or allowed to flow through a large hypodermic needle, as above.

**Complement-fixation Test.**—In 1909 Schütz and Schubert applied this method to the diagnosis of glanders. It gives excellent results, for it picks up 97 per cent. of positive cases according to Miessner and Trapp.[2] Its failures lie chiefly in the early stages of the disease, and for this reason it should be paralleled by the agglutination reaction. The combination of these two tests gives, according to Huytera and Marek[3], a percentage of 99 successful tests. The use of a mixed antigen for the detection of glanders is important.

**Agglutination Reaction.**—This reaction was first applied by MacFadyean (1896) who used the microscopic method as in the Widal reaction. Later Schütz and Miessner (1896) found the macroscopic method more practicable. In the early stages of glanders this method is most valuable. It picks up about 84 per cent. of positive cases (Anthony and Grund[4]), the failures occurring chiefly in old chronic cases.

Schütz and Miessner claim that a culture recently passed through a guinea-pig (once in three weeks) is essential for a good test fluid. In our hands cultures kept on properly made artificial media (potato-glycerin-veal-agar) do well if passed through a pig once in two months. Not every strain of B. mallei agglutinates well, consequently a suitable one must be chosen.

The *macroscopic agglutination* test may be carried out in several ways:

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[1] U. S. Dept. Agriculture, Bureau of Animal Industry, 1912, Circ. 191.

[2] Centralb. f. Bact., 1909, Band, lii.

[3] Ibid.

[4] Collected Studies, Bureau of Lab., City of New York, 1913, vii, 291.

*Incubator Method.*—The procedure of Schütz and Miessner, with slight modifications, is as follows: A forty-eight-hour acid-glycerin-agar culture of B. mallei is washed off with normal saline solution containing 0.5 per cent. pure carbolic acid. This suspension is heated at 60° C. for two hours. It is then filtered through cotton, and enough of the carbolic salt solution added to reduce it to a faintly cloudy suspension. This should be standardized by comparing it with a known test fluid, if possible, and testing it with known negative and positive sera. This test fluid will keep in the ice-box for several weeks.

The serum (active) to be tested is then made up with normal salt solution to 1:40 dilution. From this the final dilutions of 1:500, 1:800, 1:1000, etc., are made by adding 0.24 c.c., 0.15 c.c., and 0.12 c.c. respectively to 3 c.c. of the standardized test fluid in each test-tube. The tubes are well shaken and incubated twenty-four to seventy-two hours with positive and negative control sera in the same dilutions. If a reaction occurs the upper part of the fluid is clear, while a veil-like sediment is found at the bottom. A strong positive reaction (1 to 1000) may occur in twenty-four hours. A negative reaction shows the sediment in a definite "button" at the bottom of the tube and the fluid above is cloudy.

With the *centrifuge method* of Miessner and others, cited by Mohler and Eichhorn[1] the time factor is greatly reduced. The tests are incubated for a half-hour at 37° C., then centrifugalized at 1600 revolutions for ten minutes, and kept at room temperature for two hours before reading. This is done by looking down on the tubes from above toward a dark background. Indefinite reactions may be read the next day after standing at room temperature. Special tubes with perfectly rounded bottoms are essential for this method, and the dilutions are made up in only 2 c.c. of the test fluid.

The limit of agglutination (*regular methods*) in the normal horse is 1 to 500, most of the reactions occurring at 1 to 200 or 400. Since, however, some cases of chronic glanders do not react above 1 to 500, this reaction should be regarded with suspicion and checked by the complement-fixation test and eye mallein; so also reactions below 1 to 1000. Reactions of 1 to 1000 are positive, some horses running up to 1 to 2000 or 3000. In practical work dilutions higher than 1 to 1000 are unnecessary.

*Rapid Method.*—Povitzky, of this laboratory, is applying the following method of macroscopic agglutination with time-saving results. Technic: A fresh culture grown for forty-eight hours on glycerin-potato-veal agar (2.5 acid to phenolphthalein) is washed off with a small amount of sterile normal salt solution. This thick milky suspension is heated for one hour at 70° C.; it may then be kept in the ice-box for two months or more. Whenever it is to be used, normal salt solution is added until the suspension is only faintly cloudy. This test fluid must be standardized by comparison with a known standard, etc., as described under the *incubator method* of agglutination. The active serum to be tested is then diluted with normal salt solution and added in suitable amounts to 3 c.c. of the test fluid (see procedure under incubator method). The dilutions, however, are carried higher—1 : 1200, 1 : 1600, 1 : 2000 usually. The various dilutions and controls are then placed in a water-bath of 37° to 40° C. for two hours. Very active reactions can be read as early as the end of the first hour; others at the end of the second hour. The tubes are then set in the ice-box overnight and any delayed reactions may be read next morning. Although this method is only a little more rapid in time than the centrifuge method, its easier technic and lack of complicated apparatus recommend it.

In reading the rapid method tests on horses a reaction is considered as positive which has double plus through 1 to 1600; as suspicious with double plus in 1 to 1200; as doubtful with double plus in 1 to 1000. Any reactions below 1 to 1000 are considered as negative.

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[1] U. S. Dept. Agriculture, Bureau of Animal Industry, 1912, Circ. 191.

These tests should always be checked by the use of eye mallein and the complement-fixation test.

In human cases a reaction by the "rapid method" (Povitzky) of 1 to 500 and above, is considered positive. Normal human blood reacts seldom above 1 to 100, but it may reach 1 to 200 or even 400 in exceptional instances.

**Mallein Reaction.**—Mallein is like tuberculin in that it consists of glycerinated bouillon which contains the products of the growth and activity of B. mallei cultivated in it. It was discovered by Kelnig, a Russian veterinarian, in 1890. (For the preparation of the two kinds of mallein—*eye* and *subcutaneous*, see below.)

The *eye mallein reaction* is one of the most recently developed tests for glanders in animals, yet it has taken a preëminent place in diagnosis for it is the Federal test for the interstate shipment of horses. The simplicity of the application of eye mallein, the short time—twenty-four hours—required, and the comparatively easy reading of results, after a suitable experience, make it possible for the veterinarian to apply prompt tests on suspected horses.

In healthy horses the error of this test has been shown (Schnurer[1]) to be only 0.39 per cent., while in glanderous horses the test results are 88.8 per cent. positive, 3.5 per cent. negative, and 7.5 per cent. doubtful. As with the complement-fixation test the doubtful and negative reactions occur chiefly in the early stages of the disease; consequently its use alone, without the agglutination reaction, to check the complement-fixation test is subject to error unless retests at suitable intervals are planned and carried out.

*Technic of Application.*—When 2 or 3 drops of concentrated mallein are instilled into the conjunctival sac, no reaction save a slight lacrimation and congestion results in healthy horses. In glanderous animals this goes on, at the end of from five to seven hours, to profuse lacrimation, redness, edema, and the formation of pus. There may be only a drop of pus at the inner canthus of the eye, or all degrees to profuse purulent discharge. Unless pus is present, the reaction is not considered positive. The results may be recorded according to the following scheme suggested by the Bureau of Animal Industry: N = negative—eye unchanged; S = suspicious—sero-mucous discharge; P+ = positive—sero-mucous discharge with purulent flakes; P++ = positive—distinct purulent discharge; P+++ = purulent discharge with swelling of eye-lids; of both lids. There is a slight rise in temperature in those cases showing a P++++ = marked purulent discharge with swelling and gluing together marked reaction, but as the local reaction is very distinct, the tedious task of taking temperatures as in the subcutaneous method is superfluous.

Another advantage of this method over the subcutaneous inoculation, aside from its simplicity, is the fact that it can be repeated after twenty-four hours in doubtful cases; also it does not interfere with subsequent serodiagnostic tests. With very few exceptions, a second test in a glanderous horse gives a prompt reaction.

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[1] Proc. Tenth International Veterinary Congress, London, 1914.

*The intrapalpebral mallein test* is a modification of the eye mallein test. Instead of applying the mallein directly to the eye a diluted mixture is injected intradermally into the delicate skin of the lower eyelid. This test gives a local and a thermal reaction, accompanied sometimes by a general reaction.

*Directions.*—Dilute one volume of eye mallein to two or three volumes by adding a 0.5 per cent. carbolic salt solution just before using. A fold of the thin skin of the lower eyelid is grasped by the thumb and forefinger as near the margin as possible. Then the fine bore-needle of a hypodermic syringe is inserted into the dermis parallel to the margin of the eyelid midway between the inner and outer canthus. The needle should be inserted about half an inch and 0.1 c.c. of the freshly diluted eye mallein injected.

*Local Reaction.*—In the *normal* horse an inflammatory edema appears at the site of injection reaching its height at about the eighth to tenth hour and then subsides in twenty-four to thirty-six hours. This edema is neither painful nor diffuse and usually takes on a crescentic shape. The conjunctival membrane is not congested although occasionally a slight mucous or serous discharge is present. This discharge differs markedly from that of a horse which has glanders. Thermal reaction: There is no rise in temperature in a normal horse.

In a *glandered horse* the injection is followed in a few hours by a hot, painful, diffuse swelling, which may involve the upper lid and whole side of the upper part of the face—occasionally corded lymphatics, radiating from the swelling, may be noted. The conjunctival membrane is congested and a conjunctivitis with a dirty purulent discharge usually develops. The free edge of the eyelid is swollen and tends to protrude upward at the inner canthus. The height of the reaction may occur at the eighth to the tenth hour or as late as the forty-eighth or fifty-sixth hour. It may last seven days but usually only four or five days. Thermal reaction: The temperature rise may reach 105° F. and as a rule follows the local reaction in its occurrence. The *general reaction* is not marked but may involve dulness and loss of appetite.

This test has a transient effect on the serodiagnostic tests. One of its advantages, however, is that it cannot be removed or modified by an unscrupulous owner.

*Subcutaneous Injection of Mallein.*—The injection of mallein subcutaneously is one of the oldest and most reliable methods for the diagnosis of glanders. It should be applied however only after the complement-fixation, agglutination and eye mallein tests have been used, since the subcutaneous injection of mallein, as also any glanders antigen including vaccines, interferes with the serodiagnostic tests. In spite of the fact that it picks up 89 per cent. (Huytera and Marek[1]) of the positive cases, its cumbersome technic of prolonged temperature taking, the detention of the horses from work, etc., all contribute to render it unsuitable as an early test.

Before applying the subcutaneous test the temperature of the horse should be taken at least three times at intervals of three hours. If there is fever the mallein should not be given. The injection of mallein (usually about 2 c.c.) should be made about 10 P.M. In a glanderous horse there will be a local reaction and a general reaction with fever. The temperature begins to rise usually three or four hours after the injection and reaches its maximum between the tenth and twelfth hours. Sometimes the highest point is not reached until fifteen to eighteen hours after the injection. This rise in temperature is from 1.5° to 2° C. (2° to 3.5° F.). The temperature taking should be continued every two hours, beginning not later than eight to ten hours after the mallein was given. The general condition of the animal is more or less profoundly modified and the local reaction is usually very marked around the point of injection. Here, in a few hours, there appears a warm, tense and very painful swelling. Running from this will be found hot, sensitive lines of sinuous lymphatics directed toward the neighboring lymphatic nodes. This edema increases for twenty-four to thirty-six hours and persists for several days, not disappearing entirely for eight or ten days.

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[1] Centralb. f. Bact., 1909, Band, lii.

In healthy animals the rise of temperature is usually only a few tenths of a degree but it may reach 1° C. This rise should always be considered, however, in connection with the general and local reactions. At the point of injection the mallein produces only a small edematous tumor which, instead of increasing diminishes rapidly and disappears in about twenty-four hours.

**Occurrence of the Reactions of the Various Tests after Infection.**—*Agglutinins* increase above normal in four or five days and continue to rise in the early stages of the disease, diminishing as the disease becomes chronic. Specific amboceptors for the *complement-fixation* test may be demonstrated in from seven to ten days and remain during the entire course of the disease. The *subcutaneous mallein* test may, as a rule, be relied upon for diagnosis fifteen days after infection, while the *eye mallein test* is reliable twenty-one days after infection.[1]

**Effect of One Test on the Others.**—The serodiagnostic tests are influenced in three to six days after a subcutaneous injection of mallein or any glanders antigen including vaccines. The period of influence varies from six to eight weeks after the injection of mallein, and lasts three months or longer after the injection of glanders antigen or vaccines.

The injection of intrapalpebral mallein has a transient effect only on the serodiagnostic tests.

**Postmortem Lesions.**—Postmortem lesions are given above. The confirmation of the findings of all positive (or doubtful) tests by careful examinations at autopsy, injection of material into guinea-pigs, together with the study of microscopic sections, is most desirable in order to extend our present data.

**Preparation of Mallein.**[2]—Mallein for *subcutaneous injection* is produced by growing B. mallei (preferably a variety of strains) for six to eight weeks in a 5 per cent. glycerin-nutrient veal bouillon, 2.5 acid to phenolphthalein about $P_h$ 6.4. (See Chapter on Media, p. 131.) Each flask or bottle of the culture is then tested for purity by the examination of smears and cultures made on veal agar *neutral* to phenolphthalein (about $P_h$ 8 or 8.2)—a medium unfavorable to the growth of B. mallei. If pure, the broth culture is killed by steaming in the Arnold sterilizer for one hour. After sedimentation in the ice-box for a few days the supernatant fluid is filtered first through paper pulp and then through the Berkefeld filter. Carbolic acid is added to give 0.5 per cent.

For *eye mallein* the same procedure as above is followed except that after filtering the liquid through paper pulp, it is measured and then evaporated over the hot-water bath to one-tenth its volume. The viscid liquid is then sterilized by heating in the Arnold sterilizer for three-quarters of an hour. The precipitate which has formed is thrown down either by centrifugalizing, or by sedimentation in the ice-box. This latter method takes about two weeks.

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[1] Proc. Fifteenth Meeting, Am. Vet. Assn., 1913, p. 291.

[2] The methods given are those in use in the Health Department of the City of New York.

Before use both the *subcutaneous* and the *eye* mallein should be subjected to potency tests on both glandered and normal horses.

## B. ABORTUS (BANG).

This organism, first described by Bang, is the cause of contagious abortion in cattle. It is a small, pleomorphic Gram-negative bacillus which when first isolated is micro-aërophilic, becoming aërobic on cultivation. Its main interest medically is its common presence in milk and the possibility of human infection arising from this source. The presence, in the blood, of agglutinins and complement-fixing substances has been demonstrated in an appreciable number of children and in some instances of aborting women (Larson and Sedgwick,[1] Nicoll and Pratt[2]). Only once, however, has the organism been isolated from human tissues, viz., a tonsil, which cannot be considered as an infection. Whether such antibody reactions are due to intestinal absorption of the products of the bacilli contained in the milk or to the passive absorption of antibodies (Cooledge[3]) in the milk of infected cows cannot be answered. At least it does not seem that they are due to infection.

The other interest is that B. abortus when injected into guinea-pigs gives rise to lesions very similar to those of tuberculous origin (Smith and Fabyan[4]). It is evident that this may be a source of error in examining milk for the presence of tubercle bacilli by inoculation. Spontaneous infection of guinea-pigs has also occurred in a laboratory, the source of infection being inoculated pigs.

[1] Am. Jour. Dis. Child., 1913, vi, 326.
[2] Ibid., 1915, x.
[3] Jour. Med. Res., 1916, xxxiv, 459.
[4] Centralb. f. Bakt., 1912, lxi, 549; Jour. Inf. Dis., 1912, xi, 464.

# CHAPTER XXX.

# THE GROUP OF HEMOGLOBINOPHILIC BACILLI. BORDET-GENGOU BACILLUS.

## THE INFLUENZA BACILLUS.

ALL bacilli that will not grow in pure cultures without the presence of hemoglobin are called hemoglobinophilic bacilli. All bacilli having this characteristic, that have been isolated and studied, resemble each other in several other particulars. That is, they are minute, faintly staining Gram-negative rods with a tendency to bipolar staining, and the formation of delicate threads. There has not been much study of the group to separate them into distinct species or even types so their classification remains undecided. They have not been found outside of animal bodies. They have been demonstrated in several diseases both of humans and of some of the lower animals. Claims have been advanced particularly that members of the group are the cause respectively of the epidemic disease called influenza, of the disease called acute contagious conjunctivitis or "pink eye," of the conditions called trachoma. Since the recent great pandemic of "flu" the studies made of this group of organisms have altered our ideas of their relation to epidemic influenza.

Epidemics occurring periodically and possessing the same general characteristics as those classed under the name influenza can be traced back to the fifteenth century and probably existed at a much earlier date. Between epidemics endemic cases occur, but we are not certain that these are all started by the same "flu" virus. The pandemic that has just ended was probably the most widespread and severe of all.[1] The full reports on its epidemiology have not yet been completed. The epidemic before this one seemed to start in eastern Russia in the fall of 1889. It gradually spread over Europe and to America, reaching the latter country in December of that year. Since then we have had a varying number of cases diagnosed "influenza" especially numerous during the winter months. Many acute inflammations of the respiratory mucous membranes due to pneumococci and streptococci, give symptoms similar to those supposed to be due to the influenza bacillus.

The rapidity of the spread of epidemics of influenza suggested that persons were the carriers of the infection, while the location of the disease pointed to the respiratory tract as the chief location of the microorganisms.

After numerous unsuccessful attempts, during the epidemic of 1889 and succeeding years, to discover the specific cause of influenza, Pfeiffer (1892) succeeded in isolating and growing upon *blood-agar* a bacillus which he found in the purulent bronchial secretion of patients suffering from affections diagnosed as epidemic influenza, which he claimed was the cause of the disease. Canon, working at the same time, found a similar bacillus in the blood of several cases of the disease. Pfeiffer proved that in pure cultures this bacillus required hemoglobin for its growth. Though B. influenzæ was shown to have definite pathogenic powers, its specificity in epidemic influenza was not proved.

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[1] Soper: Science, 1918, n. s., xlviii, 451.

Pfeiffer's reasons for claiming that his bacillus, which has since been called Bacillus influenzæ, is the cause of epidemic influenza are as follows: (1) He found it in large numbers in every case he examined, sometimes apparently in pure cultures. (2) He found no other single variety of organism in every case. (3) He found it at the site of the lesion; that is, in the bronchial secretions in the living and, as well, in the deeper portions of the lungs after death. (4) He said that it disappeared from the secretions after the patients became entirely well. (5) He did not find it in other conditions. (6) He said that he had produced the disease irregularly in monkeys, and atypically in rabbits, and that he recovered his organism from the site of the lesion. He thus claimed to have practically fulfilled the postulates of Koch, which were supposed, at that time, to embody the proof of the specific pathogenicity of an organism.

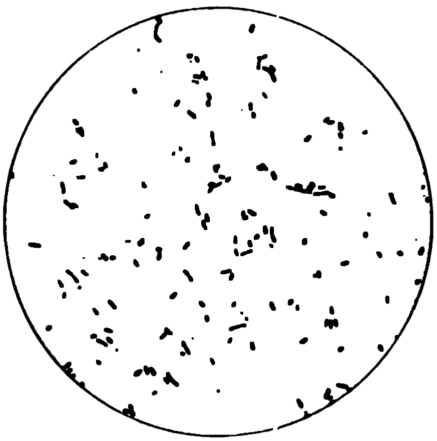

FIG. 148.—Influenza bacilli. × 1100 diameters.

We know now, however, that these "postulates" are not necessarily sufficient to prove that a given organism is specifically pathogenic; neither are all of them always essential for proof. We know that the demonstration of specific antibodies, in the host, constitutes the strongest proof of specificity, and that this must be done in those instances where similar organisms are found in other conditions, and in many apparently well people. And we know, contrary to Pfeiffer's first statement that true influenza bacilli are only found in true cases of influenza, that influenza-like bacilli, that cannot yet be differentiated from the Bacillus influenzæ by any known tests, are found in many conditions other than epidemic grip sometimes definitely as secondary infections, *e. g.*, tuberculosis, measles, pertussis, and sometimes apparently as primary infections, *e. g.*, meningitis, pink eye. And since the animal experiments were not clear-cut, and no group antibodies were demonstrated, and since other observers did not find influenza bacilli in all cases diagnosed as epidemic grip, doubts accumulated in regard to their specific action in this condition.

**Morphology of the Influenza Bacillus.**—Very small, moderately thick bacilli ($0.2\mu$ to $0.3\mu$) in thickness to $0.5\mu$ to $2\mu$ in length), usually occurring singly or united in pairs, and occasionally showing threads, are found in spreads from the sputum and young cultures. In later cultures threads may be produced in great abundance. Some strains produce threads more rapidly and abundantly than others, and others show more globoid forms. No capsule has been demonstrated.

**Staining.**—The bacillus *stains* rather faintly with the ordinary anilin colors—best with dilute Ziehl's solution (water 9 parts to Ziehl's solution 1 part), or Löffler's methylene-blue solution, with heat. Giemsa's method stains them well and brings out the polar granules which sometimes develop in these bacilli. They are not stained positively by Gram's method.

**Biology.**—An aërobic, facultative anaërobic non-motile bacillus, does not form spores; no growth occurs with most cultures below 22° C., or above 41° C. Some strains ferment the monosaccharids and a few of these produce a slight amount of gas in glucose media (Wadsworth and Wheeler[1]). Some strains produce indol (Jordan[2]).

**Cultivation.**—This bacillus is best cultivated at 37° C., on special culture media containing hemoglobin (p. 123). At the end of eighteen to twenty-four hours in the incubator at 36° C. very small colonies are developed, (1) on blood-pour agar plates, to the naked eye, as minute dew-drop colonies, and (2) on blood-drop agar plates, under the microscope (mag. 100 diam.) as small whitish areas embedded among the red-blood cells. The pneumococcus and streptococcus show a greenish coloration and rim. The influenza colonies are most typical on this plate. They often show characteristic "heapings" in the center, especially after forty-eight hours. When surrounded by blood cells, the margins of the colonies often appear irregularly scalloped, but on the edges of the blood mass the free margins of the colonies are usually entire, with an occasional indentation. It is difficult, however, to focus on a definite marginal line, as the margin seems to vanish suddenly on focussing. The third dilution plate—the blood-drop-vitamin agar plate—often shows very large colonies of influenza bacilli especially after forty-eight hours. Oleate-blood-agar (Avery, p. 128) shows very characteristic colonies in forty-eight hours, and as this medium inhibits the growth of pnuemococci and most streptococci it is a very helpful medium to use in isolating the bacillus (Fig. 149): Fishings are made and planted on glycerin-veal-blood agar (chocolate medium, p. 123). Cultures if kept moist, may remain alive for a variable time. Transplants should be made at least once a week. Pfeiffer showed that not only were the red blood cells the necessary part of the blood needed for the growth of the influenza bacillus but that it was the hemoglobin in the cells that was the essential element.

Though in pure cultures this organism will not grow without hemoglobin, in the presence of certain other organisms it will grow abundantly for successive culture generations without blood. Meissner and others found that it would grow well with members of the diphtheria group, and with the pyogenic cocci. We have found, contrary to others, that staphylococci grown in mass cultures tend to inhibit the bacilli.

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[1] Personal communication.

[2] Jour. Am. Med. Assn., 1919, lxxii, 1542.

**Resistance and Length of Life.**—The influenza bacillus is very sensitive to desiccation; a pure culture diluted with water and dried is destroyed with certainty in twenty-four hours; in dried sputum the vitality, according to the completeness of drying, is retained from twelve to forty-eight hours. It does not grow, and soon dies in water. In blood-bouillon cultures at 20° C. it retains its vitality for several weeks. In moist sputum it is difficult to determine the duration of its life, since the other bacteria overgrow and make it impossible to find it. It probably can remain alive for at least two weeks. The bacilli are very readily killed by chemical disinfectants, and succumb to boiling within one minute and to 60° C. within five minutes.

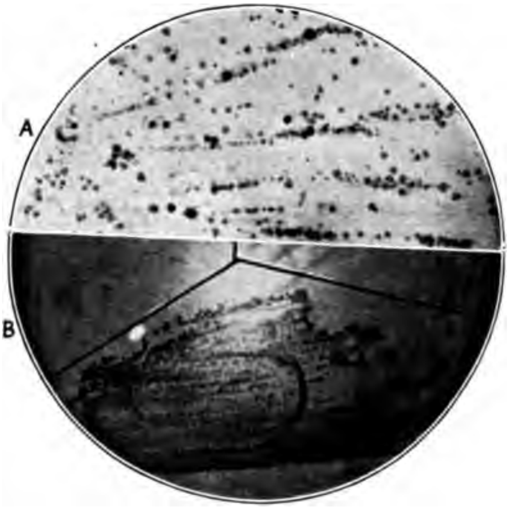

FIG. 149.—Bacillus influenzæ from throats grown on oleate-hemoglobin agar. *A*, throat culture from a normal person showing large colonies of B. influenzæ; thirty-six hours growth at 37° C. (only half plate shown). *B*, corresponding sectors are planted with the same organisms as in *A*, and show enhanced growth of B. influenzæ in the lower sector and complete inhibition of growth of pneumococcus and Streptococcus hemolyticus (only half plate shown). (Pritchett and Stillman.)

**Effect on Animals.**—The bacillus of influenza is definitely though moderately pathogenic for some animals, especially the rabbit, and several observers have reported that such pathogenic power (from one blood-agar slant to $\frac{1}{10}$ of a slant) may be decidedly increased by successive passage through the susceptible animal. We have not been able to corroborate this. Guinea-pigs and mice are also quite susceptible to some strains. Pure, abundant cultures are always obtained from the heart after intraperitoneal or intravenous inoculation. Wollstein has been able to produce cerebrospinal meningitis in the monkey by subdural inoculation of a strain from meningitis. Toxic products cause toxic symptoms in animals only when inoculated in comparatively large quantities. (Parker,[1] Huntoon.[2])

**Immunity.**—Short immunity may be established after an attack, though in some cases animals seem to be more sensitive to a second attack. The difference in strains may account for this. That rabbits may be hyperimmunized is shown in the process of obtaining antibodies.

**Antibody Production.**—That the various strains of hemoglobinophilic bacilli should be of one type from the standpoint of antibody reactions could scarcely be conceived, but owing to the apparent inability to get clear-cut results in animals the question remained until recently an open one. Wollstein claimed a relationship between the strains that she isolated from meningitis and pneumonia, but she was only able to get low dilution agglutinations and did not make any absorption tests, neither did any one else.

**Agglutination.**—When we tested a very large number of strains isolated during the recent pandemic for specific agglutinins by the absorption test (see Chapter XIII) we were surprised to find that practically each strain stimulated the production of abundant agglutinins, but the specific agglutinins were usually absorbed only by the serum strain, very exceptionally by another. That is, similar strains were very infrequently encountered. This evidence of multiple strains from actual cases seems to be absolutely against the influenza bacillus being the cause of the pandemic, since a microbal pandemic must be due to later generations of the original virulent strain and it is not probable that we could miss a pandemic strain in so many cases or that the bacilli would become changed rapidly in the patient in their ability to produce specific agglutinins.

**Complement-fixation.**—Specific antibodies may be obtained in most rabbits, which definitely fix complement. The greatest difficulty in demonstrating the phenomenon of complement-fixation with this group of organisms, is the preparation of suitable antigens. We have found that with the use of only small quantities of heated blood in the medium and by employing the method of shaking the cultures and then incubating overnight to help the autolysis, very satisfactory antigens have been obtained. Definite groups among these bacilli have not been demonstrated by this reaction.

**Pathogenicity for Man.**—The invasion of the body by the influenza bacillus is more widespread than was earlier supposed. It is probably more frequently secondary than primary. Very frequently the influenzal process invades portions of the lung tissue. In severe cases a form of pneumonia is the result, which is lobular, hemorrhagic and purulent in character and accompanied by symptoms which may be somewhat characteristic for influenza, or, again, almost identical with bronchopneumonia due to the pneumococcus. The walls of the bronchioles and alveolar septa become densely infiltrated with leukocytes, and the spaces of the bronchial tubes and alveoli become filled. The influenza bacilli are found crowded in between the epithelial and pus cells and also penetrate the latter. There may be partial softening of the tissues or even abscess formation. Bacilli are found in fatal cases to have penetrated from the bronchial tubes not only into the peribronchitic tissue, but even to the surface of the pleura, and in a few cases they have been obtained in pure cultures in the pleuritic exudation.

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[1] Parker: Jour. Am. Med. Assn., 1919, lxxii, 476.

[2] Huntoon, and Hannum: Jour. Immun., 1919, iv, 167.

**Presence in Other Parts of the Body.**—Influenza bacilli are found at times in the blood during the early days of an acute infection, while there is marked fever and sometimes in severe cases in young children a septicemia develops before death. In some outbreaks they are found much more than in others. The difference depends again probably on there being different varieties. They are found at times in otitis media accompanying influenza and have been found in cases of meningitis (Wollstein[1]), in many cases of acute and subacute conjunctivitis (Williams[2]), frequently in sinusitis acute and chronic, and in several cases of peritonitis, appendicitis, and cystitis.

*Toxic Effects.*—The general, cerebral, gastric, and other symptoms produced are usually due to the absorption of the toxic products and of the specific organism, these poisons being particularly active in their effects on the central nervous system.

**Presence of Influenza Bacilli in Chronic Influenza and in Tuberculosis.**—Ordinarily influenza runs an acute or subacute course, and not infrequently it is accompanied by mixed infections with the pneumococcus and streptococcus. Pfeiffer was the first to draw attention to certain chronic conditions depending upon the influenza bacillus. Bacilli may be retained in the lung tissue for months at a time, remaining latent a while, and then becoming active again, with a resulting exacerbation of the disease. Consumptives frequently carry influenza bacilli for years and are particularly susceptible to attacks of influenza. Williams, in the examination of sputa in cases of pulmonary tuberculosis, found abundant influenza bacilli to be present in a large proportion of the samples of sputum from consumptives, and this not only in winter but also in the summer, when no influenza was known to be present in New York. Taken together with results elsewhere, this indicates that at all times of the year many consumptives carry about with them influenza bacilli.

*Influenza Bacilli in Other Diseases and in Normal Persons.*—Persons suffering from bronchitis and all other diseases affecting the mucous membranes, especially of the upper respiratory, tract may harbor very many of these hemoglobinophilic bacilli. (Lord, Davis, ourselves and others). They may be found quite frequently also in apparently healthy persons.

**Bacteriological Diagnosis.**—In acute respiratory infections the bacilli are found as a rule *most abundantly in the nasopharynx*, therefore cultures should always be taken from this area as well as from the sputum. Material from the nasopharynx is collected on a swab, which is introduced, protected by a West tube and planted on media similar to that given below.

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[1] Jour. Exp. Med., 1911, xiv, 73.

[2] Jour. Inf. Dis., 1914, xiv, 261.

**Examination of Sputum for Influenza Bacilli.**—1. Sputum coughed from the deeper air passages and not from throat scraping should be used.

2. The sputum should be expectorated into a sterile bottle, which should then be placed immediately in cracked ice to transport to the laboratory.

3. Blood-drop-agar plates should be made by placing a drop of fresh rabbit or horse blood, obtained aseptically, on the side of a hardened vitamin-blood-agar plate (see p. 124). Blood-pour plates and oleate-blood-agar (p. 129) plates should also be used.

4. One of the more solid masses of the sputum should be taken from the bottle with sterile forceps and placed on a plain agar plate. A small portion of this mass should be separated with a sterile platinum needle and drawn through the blood on the blood-agar plate out in different directions. The larger part of what is left of this small portion is then placed in a similar manner over a second blood agar, and from this to a third, sterilizing the needle between the transfers. Similar seedings should be made on the other blood-media plates. The plates should be placed in the incubator at about 36° C. for twenty-four to forty-eight hours.

Similar cultures should be made from the nasopharyngeal swabbings.

5. After the plates are planted two smears should be made from the sputum, one stained by Gram and the other by weak carbol-fuchsin.

6. After twenty-four to forty-eight hours the plates are examined under low power. The influenza colonies use up the hemoglobin, and in parts of the blood-agar plate where the blood is of right thickness such colonies show as almost clear white areas surrounded by the red blood. With a higher power (No. 6 or 7 objective), if such areas seem to be made up of fine indefinite granulations, they are practically sure to be influenza colonies. Other characteristics have already been given.

7. Fishings from the influenza-like colonies should be planted on chocolate-agar-tubes, and if, after twenty-four hours in the thermostat, the resulting growth should consist of influenza-like organisms, plantings should be made on plain agar. The first generation on plain agar may show slight growth because of the blood carried over from the original tube, but the second generation should show no growth if the organism is the influenza bacillus.

**Other Bacilli Resembling the Influenza Bacillus.**—There are a number of bacilli which differ slightly in morphology and growth in culture from the characteristics of the typical influenza bacillus. These were grouped under the name "*pseudo-influenza bacilli.*" But so far there have been shown no characteristics distinct enough to separate these hemoglobinophilic organisms into other than "strains" or "varieties" of the one species, B. influenzæ. For example, the influenza-like bacilli found first in whooping-cough by Jochmann and others, Müller's "trachoma bacillus," Koch-Weeks' bacilli, the bacilli found by Cohen in meningitis, and those reported occasionally in other parts of the body—all of them seem to be so closely related that they should be considered one species or, at the most, varieties of one species until more specific characteristics can be demonstrated. With the more minute cultural and serologic studies that are being carried on since the great pandemic differences are sure to be demonstrated.

**Relation of the Clinical Symptoms to the Bacterial Excitant.**—There is no doubt that several infections are also included under the clinical forms of influenza, during interpandemic times and during epidemics of acute respiratory infections, irregular types of lobar pneumonia, and cases of bronchitis frequently have symptoms so closely alike that the nature of the bacteria active in the case is very frequently different from that supposed by the clinician. Thus in four consecutive autopsies examined by the writers the influenza bacillus was found almost in pure culture in one case believed, from the symptoms, to be due to the pneumococcus, and entirely absent in two of the three believed to be due to it. Except for these examinations the clinician would be of the opinion that he had clearly diagnosed bacteriologically the cases, while in fact he had been wrong in three of the four.

The striking symptoms in acute respiratory diseases are frequently due more to the location of the lesions than to the special variety of organisms producing them. In epidemics of influenza there are, of course, many cases which, on account of their characteristic symptoms, can be fairly certainly attributed to one type of organism. This is especially true in such a pandemic as the one just ended, where so many cases came down at the same time or in rapid succession, with such similar symptoms. But we must remain far from certain of the entity of the epidemics called influenza until we have proof of a specific cause.

**Recent Studies on the Specific Microbal Cause of Epidemic Influenza.** —Among the many reports on the bacteriology of the recent pandemic, three claims for a specific etiological agent stand out in importance above the others: (1) That for a special streptococcus; (2) the claim for the influenza bacillus; (3) the claim for a filtrable virus.

*Evidence in Regard to Streptococci being the Initiating Specific Cause.*—Gram-positive green-producing cocci in twos and chains of varying lengths have been reported by numerous observers as the cause of "flu" from the time of the 1889 pandemic, but the only evidence given in favor of them up to the present pandemic has been certain cultural similarities and the presence of apparently similar forms in large numbers in sputum and lungs of the majority of cases. During the early days of this pandemic, reports came from Europe that Gram-positive cocci were found but no specific evidence was given. Here in this country Mathers (reported by Tunnecliff, November, 1918) found in 87 per cent of 110 cases at Camp Meade a "culturally characteristic green-producing streptococcus." Tunnecliff tested the opsonic power of the serum of four influenza patients and ten pneumonia cases against three strains and found increased opsonins as the patients recovered in the four influenza cases and in two of the pneumonias. Four more patients were tested in Chicago, with positive results. Two control strains (Micrococcus catarrhalis and Streptococcus hemolyticus) were used. These results are suggestive, but too few strains, cases and controls were used.

Rosenow claims to have found a similar streptococcus, though he describes the cultural characteristics somewhat differently, emphasizing their variability. He states that several organisms have in this pandemic shown selective affinity for the lungs and chief among them is this streptococcus. He bases his claim for specificity upon the production of similar lesions in guinea-pigs when inoculated into the trachea and on the production of agglutinins in a horse, but he has not controlled his agglutination tests sufficiently by absorption of agglutinins, and until that is done nothing can be said for specificity of type.

*Evidence in Regard to the Influenza Bacillus being the Initiating Specific Cause.*—Here, again, we must show identity of strain or we add no new evidence in favor of the influenza bacillus being the exciting cause of the pandemic. When we began our studies on the significance of B. influenza in this pandemic and found these bacilli in practically 100 per cent. of the cases, we immediately started investigations to show identity of strains.

As we have shown above, agglutinin tests controlled by absorption of agglutinins have given such clear-cut negative results that they have practically overthrown the evidence in favor of the influenza bacillus being the specific inciting cause.

*Evidence in Regard to a Filtrable Virus being the Initiating Cause.*—Here too we have to consider the question of differentiating between a common invader of the respiratory tract and a pandemic strain.

Foster, several years ago claimed that common colds are due to a filtrable virus. Some English observers have reported a filtrable virus in meningitis and other diseases. Three Frenchmen early reported a very few experiments indicating the possibility of a filtrable virus being present in this pandemic. Rosenow and Keegan had negative results in very small series of human inoculations. Then Dr. McCoy and his co-workers carried on two series of experiments with negative results, though these were made too late in the pandemic to draw positive conclusions.

Two groups of Englishmen have reported positive cultures (globoid bodies) by "Noguchi's method" and positive results in monkeys and some other animals but so far their statements are too general and their experiments too few to draw any conclusions.

These studies on the specific initiating cause of the 1918–1919 pandemic of "flu" may be summed up as follows: (1) The influenza bacillus is probably not the initiating cause of the pandemic; (2) too little minute work has been done on streptococci and on a filtrable virus to give evidence for or against a member of either group being the specific etiologic factor in the pandemic.

**Serum Therapeutics and Vaccine Treatment.**—These are discussed in Part III.

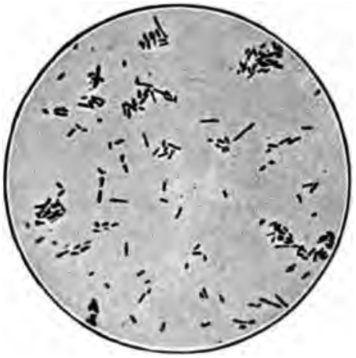

FIG. 150.—Koch-Weeks' bacillus from "pink-eye" — third generation. × 1000 diameters. (Weeks.)

FIG. 151.—Secretion of mucopus from conjunctiva in "pink-eye." × 1000 diameters. (Weeks.)

## INFLUENZA-LIKE BACILLI IN CONJUNCTIVITIS (INCLUDING TRACHOMA).

**The Koch-Weeks' Bacillus.**—This bacillus was first observed by R. Koch in 1883 while making certain investigations on inflammation of the eye occurring during an epidemic of cholera in Alexandria. It was later, in 1887, more specifically described by Weeks[1] in New York.

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[1] New York Med. Rec., 1887, xxvi, 571.

Weeks obtained it in cultures growing with the xerosis bacillus from cases of "pink-eye," or acute contagious conjunctivitis. Morax stated that he was able to obtain pure cultures on hemoglobin-free media only until the third culture generation. Others state that on human serum or hydrocele fluid they have obtained growths for many generations. Kamen concludes that it is a strict hemoglobinophile. Our studies led us to agree with this last conclusion. The successive cultures obtained with some sera are probably due to the presence of hemoglobin in amounts too small to be easily detected, but large enough to allow growths of hemoglobinophilic bacilli. The few differential points claimed between this bacillus and influenza bacilli do not hold (Williams) and so the question of their relationship is not settled. The general description of the influenza bacilli applies equally to both organisms.

**Other Microörganisms in Conjunctivitis.**—Many organisms are found in diseases of the eye, but few of these present evidence of specific etiology.

MORAX-AXENFELD BACILLUS.—In certain subacute inflammations of the conjunctiva, especially noticeable about the angle of the eyes (angular conjunctivitis), Morax (1896) and later Axenfeld found a bacillus which they consider the cause of the disease.

*Morphology.*—Short (about 2µ long), thick, non-motile bacilli, generally in twos, but sometimes single or in short chains. They take the ordinary stains easily, but are decolorized by Gram's stain.

*Cultures.*—At 37° C. the bacilli produce a delicate growth on media containing blood or serum. Later cultures grow slightly on nutrient veal agar. They grow slightly, if at all, at room temperature.

Upon serum agar, they form delicate grayish colonies.

Upon Löffler's blood serum after twenty-four to thirty-six hours the growth appears as an indentation of the medium due to liquefaction. This liquefaction continues slowly for a variable time.

In ascitic broth cloudiness is produced within twenty-four hours.

*Pathogenicity.*—Lower animals so far have shown themselves refractory. In human beings inoculations of pure cultures have produced subacute conjunctivitis.

BACILLUS OF ZUR NEDDEN.—This bacillus has been found by zur Nedden in certain ulcers of the cornea and is supposed to be the etiological factor in this disease.

*Morphology.*—Small (usually less than 1µ long) rather slender single, sometimes slightly curved, non-motile bacilli. They may occur as diplobacilli, but they do not form chains.

They stain easily, sometimes faintly at ends. They are decolorized by Gram.

*Cultures.*—They are easily grown on all laboratory media. Upon agar, within twenty-four hours they produce rounded, raised, translucent, slightly fluorescent colonies, which are more or less confluent and, under the low power, are rather coarsely granular. Upon *potato* they form a thick, yellowish growth. *Milk* is coagulated; *gelatin* is not liquefied; dextrose is fermented without gas. No indol is produced.

*Pathogenicity.*—Pure cultures inoculated in the cornea of guinea-pigs produced ulcers.

TRACHOMA.—Many studies have been made on the etiology of trachoma (progressive follicular inflammation of the conjunctiva followed by cicatrization) and allied conjunctival affections. Halberstädter and Prowazek (1907) state that the cause of trachoma is a small germ which grows in a characteristic way in the conjunctival epithelial cells. The organism itself, they say, is so small that at first it cannot be seen, only the mantle which it produces is demonstrable. This stains blue with Giemsa, and as the organisms grow in bunches, one sees at first in the neighborhood of the nucleus only a bunch of small, blue, coccus-like bodies. The organism finally appears as a minute red granule within the blue body. As it continues to increase in numbers and size, the blue mantles finally disappear, leaving a mass of small rounded or slightly elongated red bodies. The bodies are only found in the early acute cases. Prowazek named them *Chlamydozoa* on account of their mantle, and thinks they should occupy a place between bacteria and protozoa.

Our studies[1] given below show that there is similarity between these inclusions and nests of growing hemoglobinophilic bacilli.

The Koch-Weeks' bacillus has been frequently reported as occurring in trachomatous eyes, from the time of Koch (Collins, Morax, Müller, etc.). Markus states that the Koch-Weeks' bacillus is the cause of "Schwellungskatarrh" (our papillary conjunctivitis). Müttermilch goes further in declaring that "often repeated infection with the same microörganism, *e. g.*, bacillus of Koch-Weeks, produces a series of exacerbations on an already inflamed conjunctiva and finally produces the picture of trachoma."

Müller, who isolated hemoglobinophilic bacilli from the largest series of trachoma cases reported, had positive results chiefly in his "acute trachoma" cases. Müller thought at first that his bacillus was the cause of trachoma, but others thought that it was an influenza bacillus and had nothing specifically to do with trachoma (zur Nedden, Morax and others).

We, however, agree partly with both workers. We have demonstrated the continued presence of hemoglobinophilic bacilli in cases showing successively acute, subacute and chronic inflammation of the conjunctiva and their increase in numbers during acute exacerbations of chronic cases. We have also shown that the bacilli found in cases of "pink-eye" are indistinguishable, thus far, from hemoglobinophilic bacilli found in the chronic cases. Furthermore, we have pointed out a relationship between hemoglobinophilic bacilli and trachoma inclusions.

In studying closely the morphology in cultures of these bacilli, we were struck by the fact that they frequently grew in more or less dense clumps of extremely minute and irregular coccoid forms. This led us to the conclusion that possibly they form the Prowazek inclusions found in trachoma, and when we found that these bacilli and the inclusions were found coincidently and repeatedly in so many cases diagnosed as acute papillary trachoma (called by us papillary conjunctivitis), the possibility became a probability and we proceeded to study the morphology of the cultures more minutely.

The morphology varies somewhat with the age of the inoculated culture, the number of culture generations, the kind of medium and the strain. After forty-eight hours' incubation the forms become somewhat more irregular. Then in three days most of the bacilli have become extremely minute, many showing only as reddish granules (the "elementary bodies of Prowazek"), while scattered through the culture are swollen spheroidal bodies taking a fainter clear blue stain (the larger "initial bodies of Prowazek"), in some of which are minute reddish granules (more of the "elementary bodies of Prowazek"). A number of irregular light blue bodies are also scattered through the culture. Where the bacteria are densely grouped more red granules may appear in the center of the group than at the periphery and more blue bodies at the periphery than in the center. In short, all of the changes described by Prowazek and others as characteristic of trachoma inclusions are seen in the growing cultures of these hemoglobinophilic bacilli.

Similar day-to-day studies were undertaken with the other types of bacteria found most frequently in the eyes diagnosed as trachoma, *e. g.*, streptococci, staphylococci, gonococci, a minute Gram-negative, non-hemoglobinophilic bacillus not before described, found in a few cases of papillary conjunctivitis, xerosis bacillus and Micrococcus tetragenus, but in none of these varieties except the gonococcus were similar changes found in the same marked degree.

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[1] Jour. Inf. Dis., 1914, xiv, 261.

The fact that the gonococcus cultures showed such definite appearances similar to the trachoma inclusions led us to make a special study of a series of ophthalmia neonatorum cases.

In all of the inclusion cases where gonococci are found, apparent transition forms between gonococcus and inclusion are very evident, and we find from a further microscopic study of these slides that the inclusions on the whole present certain characteristics different from those found in our series produced, according to our hypothesis, by nests of growing hemoglobinophilic bacilli.

From this comparative study of "inclusions" and cultures we have reached the following conclusion:

In many cases of "papillary conjunctivitis" and a certain number of cases of opthalmia neonatorum, as well as in a certain number af cases of inflammation of the mucous membranes of other parts of the body (*e. g.*, vagina, urethra), the trachoma inclusions found are due to one or more varieties of bemoglobinophilic bacilli; in a certain number of cases of gonorrheal ophthalmia as well as in gonorrheal inflammation of the mucous membranes of other parts of the body, the trachoma inclusions are due to the gonococcus. According to one of the later reports of Leber and Prowazek and the reports of Noguchi and Cohen,[1] certain inclusion conjunctivitis cases may be caused by microörganisms other than the two mentioned above.

## THE BORDET-GENGOU BACILLUS (B. PERTUSSIS.)

In 1906 Bordet and Gengou[2] announced that they had discovered the etiological factor of whooping-cough to be a small bacillus found in predominating numbers in whooping-cough sputum. To this organism they gave the name Bacillus pertussis. Their claim that this bacillus is the real cause of whooping-cough, they based upon their results with the complement-fixation test which they had been first to describe.

**Morphology.**—The pertussis bacillus is a short, oval rod, varying in size from about $0.2\mu$ to $0.3\mu$ in diameter and from $0.5\mu$ to $2\mu$ in length. It occurs singly, sometimes in twos joined at the ends, and very exceptionally in short chains.

**Motility.**—It is non-motile.

**Staining.**—The pertussis bacillus is decolorized by Gram's method. It is stained faintly by the ordinary anilin dyes. Bipolar staining is demonstrated very well by Gram's method and by toluidin blue (p. 78).

**Cultivation.**—The pertussis bacillus grows best at 35° C., to 37° C. It grows slowly at room temperature.

It is aërobic, facultative anaërobic. When first isolated in pure culture it grows only upon the glycerin-potato-blood-agar, recommended by Bordet and Gengou (p. 120). In later generations it grows more or less capriciously, upon the ordinary culture media.

**Isolation.**—The pertussis bacillus is isolated from sputum with difficulty, owing to the fact that in cultures it is frequently overgrown by other organisms found in the respiratory tract. The sputum should be collected from the patient during the early stage of the disease, best in the first week. The thick grayish portion of the sputum should be selected for the culture. This material is streaked over the surface of a plate of Bordet-Gengou medium. The plates are incubated at 35° C. to 37° C. In forty-eight hours to three days very minute, discrete, elevated colonies appear. When these colonies occur in abundance, the blood at their periphery, is lighter red. In pure cultures this lighting of the blood is marked and may appear as hemolysis.

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[1] Arch. Ophthal. 1911, xl, No. 1.

[2] Ann. de l'Inst. Past., 1906, xx, 573.

**Identification.**—(*a*) **Differential Diagnosis by Culture.**—There are other bacilli occurring in whooping-cough sputum so closely resembling the pertussis bacillus in morphological and staining characteristics that they cannot be distinguished in smears. These bacilli, however, can be differentiated by their growth upon various culture media. The most important of these organisms is the influenza bacillus, which never grows alone without the presence of hemoglobin in the culture medium. There is frequently found a Gram-negative bacillus which makes a profuse growth upon all media from the first generation in pure culture. The following table gives the chief points of differentiation:

| | Growth on Bordet-Gengou plates. | Bordet-Gengou slants. | Coagulated horse-blood slants. | Glycerin-ascites-agar slants. | Plain agar slants. |
|---|---|---|---|---|---|
| B pertussis . . . | Lightens the medium | First generation: in pure culture abundant non-spreading. moist growth in twenty-four hours. | After several generations: abundant tenacious growth in forty-eight hours. | After several generations: abundant tenacious growth in forty-eight hours. | After several generations: tenacious growth occurs slowly. |
| B. influenzæ . . . | Darkens the medium | First generation: delicate growth | First generation: abundant moist spread in twenty-four hours | Never grows | Never grows. |
| Intermediate group of Gram-negative bacilli | Markedly lightens the medium | First generation: abundant moist spread in twenty-four hours | First generation: profuse moist spread in twenty-four hours | First generation: profuse moist spread in twenty-four hours | First generation: profuse moist spread in twenty-four hours. |

(*b*) **By Agglutination.**—Agglutinins are easily obtained in rabbits for the B. pertussis and by absorption tests we[1] have shown that all strains tested by us belong to the same species. It is easy therefore to identify any strain isolated. It is not so easy, however, to diagnose a human case of whooping-cough by this method since agglutinins are not produced to any extent in the natural affection.

(*c*) **Complement Fixation.**—Bordet and Gengou regarded the complement fixation as the main support for their claim of the specificity of their organism. They report positive complement fixation in the majority of human cases tested by them. Other investigators, however, have reported negative tests of complement fixation.

The irregular reports given of the complement-fixation test in human beings may be accounted for by different methods used without making comparative studies and sufficient controls. The question of the best method of making the complement-fixation test is still under experiment

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[1] Povitzky: Arch. Int. Med. 1916, xvii, 279.

(Chapter XIV). Different observers have used different methods of preparing both antigen and serum and have employed different hemolytic systems. In experimental animals, specific antibodies are produced which give a positive complement-fixation test.

**Pathogenicity.**—The specific pathogenicity of the pertussis bacillus for man still lacks proof, though the evidence is strongly in favor of this organism being the cause of pertussis. Several investigators have reported its pathogenicity for monkeys, dogs, rabbits, and guinea-pigs.

Mallory[1] claims that a certain number of bacilli between the cilia of the epithelial cells in the trachea and bronchi constitute the specific lesion. He claims that he has fulfilled Koch's laws by the finding of these bacilli in experimental animals in the same situation and by the recovery of the culture from these animals. He acknowledges, however, that his results in animals are complicated by the fact that these animals are frequently infected by the Bacillus bronchisepticus (accepted as the cause of distemper in dogs) which is morphologically similar to the pertussis bacillus and that it apparently has the same power (as Theobald Smith and others have pointed out) to cling to the cilia of the epithelial cells in the respiratory tract. In human beings Mallory states, judging from the examination of hundreds of controls, only pertussis cases showed this lesion.

The results of treatment by vaccine are given in Part III.

## THE BACILLUS OF SOFT CHANCRE.

This bacillus was first specifically described and obtained in pure culture by Ducrey in 1889. An experimental inoculation is followed in one or two days by a small pustule. This soon ruptures and a small round depressed ulcer is left. About this other pustules and ulcers develop which tend to become confluent. The base of the ulcer is covered with a gray exudate and its edges are undermined. There is no induration such as in the syphilitic chancre. The secretion is seropurulent and very infectious. The process usually extends to the neighboring lymphatics, which become swollen and may result in abscesses. These are known as "buboes."

**Morphology.**—About 1.5$\mu$ long and 0.4$\mu$ thick, growing often in chains and in cultures, sometimes twisted together in dense masses.

It *stains* best with carbol-fuchsin, and shows polar staining. It is Gram-negative.

**Cultural Characteristics.**—The following method of cultivation has given the best results: Two parts of liquefied agar at 50° C. are mixed with one part human, dog, or rabbit blood. The blood from the cut carotid of a rabbit may be allowed to run directly into the agar tube, to which the pus from the ulcerated bubo is then added in proper proportion, and the whole placed in the incubator at 35° C. The pus may be obtained by puncture and aspiration from the unbroken ulcer, or if the ulcer is already open it is first painted with tincture of iodin and covered with collodion or sterile gauze. After twenty-four to forty-eight hours, some pus having collected under the bandage, inoculations are made from it. The bacillus grows well also in uncoagulated rabbit-blood serum or in condensation water of blood agar. In twenty-four to forty-eight hours, on the surface of the media, well-developed, shining, grayish colonies, about 1 mm. in diameter may be observed. The colonies remain separate, but they increase in numbers after further transplantation. The best results are obtained when the pus is taken close to the walls of the abscess. Smears show isolated bacilli or short parallel chains with distinct polar staining.

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[1] Mallory and Horner: Jour. Med. Res., 1912, xxvii, 115; 1913, p. 391.

The characteristic soft chancre is produced in man after inoculation of cultures. Animals in general cannot be infected, but positive results. have been obtained with monkeys and cats.

The organisms are especially characteristic in the water of condensation from blood agar, the bacilli being thinner and shorter, with rounded ends; sometimes long, wavy chains are found. In rabbit-blood serum at 37° C. a slight clouding of the medium is produced and small flakes are formed, consisting of short bacilli or moderately long, curved chains, showing polar staining.

The bacillus lives several weeks on blood agar at 37° C., but it soon dies in cultures on coagulated serum. All other ordinary culture media so far tried have given negative results, and even with the media described, development is difficult and often fails entirely.

The chancre bacillus possesses but little resistance to deleterious outside influences. Hence the various antiseptic bandages, etc., used in treatment of the affection soon bring about recovery by preventing the spread of inoculation chancre.

## CHAPTER XXXI.

# MICROÖRGANISMS BELONGING TO THE HEMORRHAGIC SEPTICEMIA GROUP.

A NUMBER of bacilli of similar characteristics have been described as causing certain infectious diseases of lower animals, marked by the appearance of hemorrhagic areas throughout the body (hemorrhagic septicemia of Hueppe). The bacilli are short, non-motile, non-spore-bearing organisms. They exhibit bipolar staining and are Gram-negative. They do not liquefy gelatin. They are found in rabbit septicemia, fowl cholera, swine plague, and a similar disease in cattle. The bacillus of bubonic plague seems to be closely related to the bacteria of this group.

### BACILLUS OF CHICKEN CHOLERA.

(*Bacillus Avisepticus.*)

In 1880, Pasteur carried on some fundamental studies on the bacillus of chicken cholera. The bacillus was isolated from a widely disseminated acute disease of fowls and smaller birds.

**Characteristics.**—It is a short (0.5-1$\mu$ long) non-motile bacillus with marked polar staining. In general, its characteristics are similar to those of other members of this group.

**Pathogenicity.**—Pure cultures are very pathogenic for chickens and rabbits, less so for sheep, pigs and horses. Chickens are infected even by feeding minute amounts. A septicemia is produced which is rapidly fatal.

### BACILLUS OF SWINE PLAGUE.

(*Bacillus Suisepticus.*)

This organism is morphologically and culturally similar to the B. avisepticus. It differs in pathogenesis in that it produces naturally a disease of swine, characterized by a more or less chronic bronchopneumonia followed by septicemia. The gastro-intestinal tract is not markedly affected. The disease is generally fatal in young pigs. Thomsen[1] has reported 3 cases of infection in man.

The "bacillus of hog cholera" (see p. 393) may often be found as a mixed infection with the B. suisepticus.

### BACILLUS OF BUBONIC PLAGUE (BACILLUS PESTIS).

Historically we can trace the bubonic plague back to the third century. In Justinian's reign a great epidemic spread over the Roman empire and before it terminated destroyed in many portions of the country nearly 50 per cent. of the people. The fourteenth century saw the whole of Europe stricken with this "black death." Europe and America have of late been practically free, but in India the disease still breaks out in all its horrors so that at the present time over 500,000 persons die annually from it. Among the most fatal forms of infection is that of the lungs. Pneumonic cases are not only very serious, but they readily spread the infection. The bacillus exciting the disease was discovered simultaneously by Kitasato and Yersin (1894) during an epidemic of the bubonic plague in China. It is found in large numbers in the seropurulent fluid from the recent buboes characteristic of this disease and in the lymphatic glands; more rarely in the internal organs except in pneumonic cases when the lungs and sputum contain immense numbers. It occurs in the blood in acute hemorrhagic cases and shortly before death. It also occurs in malignant cases in the feces of men and animals. The bacillus, as we have stated, is closely allied to the hemorrhagic septicemia group.

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[1] Hospitalstidende, 1918, lxi, 203.

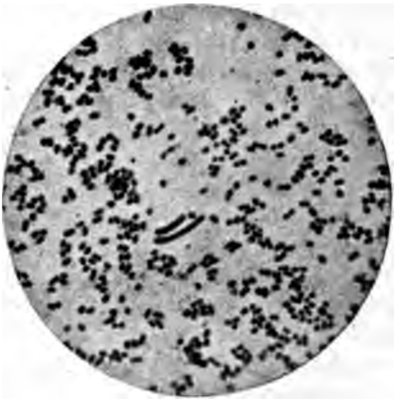

FIG. 152.—Bacillus pestis from agar culture. × 1100 diameters.

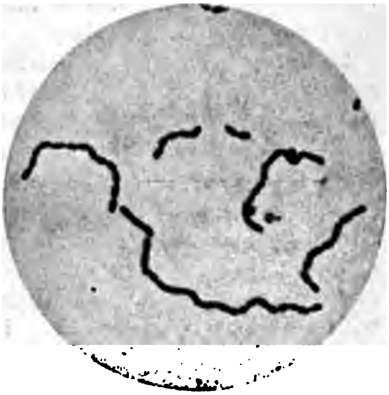

FIG. 153.—Bacillus pestis from bouillon culture. × 1100 diameters.

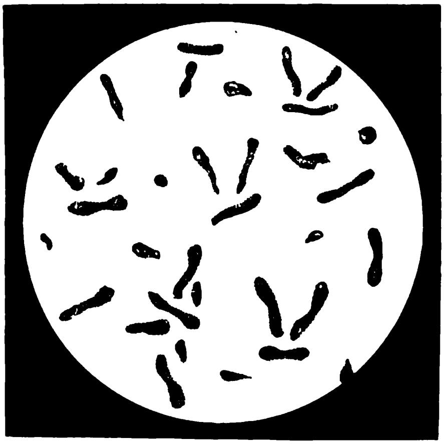

FIG. 154.—Involution forms on salt agar. (Kolle and Wassermann.)

**Morphology.**—The bacilli in smears from acute abscesses or infected tissues are, as a rule, short, thick rods with rounded ends. The central portion of the bacillus is slightly convex. The bacilli are mostly single or in pairs. Bacilli in short chains occur at times. The length of the bacilli varies, but on the average is about

1.6μ (1.5μ to 1.7μ), breadth 0.5μ to 0.7μ. Besides the usual oval form, the plague bacillus has many exceptional variations which are characteristic of it. These pleomorphic forms are especially marked on 3 per cent. salt agar. In smears, especially from old buboes, one looks for long bacilli with clubbed ends (similar to involution forms, Fig. 154), yeast-like forms, and bladder shapes. Some of these stain faintly.

**Staining.**—They *stain* readily wih the ordinary anilin dyes, and especially well with methylene blue, the ends being usually more deeply colored than the central portion; they do not stain by Gram's method.

**Biology.**—An aerobic, non-motile bacillus. Grows best at 30° to 35° C. Does not form spores. Grows on the usual culture media, which should have a slightly alkaline reaction. Does not liquefy gelatin. Grows well on *blood-serum* media. It grows rapidly on *glycerin agar,* forming a grayish-white surface growth. The bacilli appear, as a rule, as short, plump, oval bacilli, but a few present elongated thread forms which are very characteristic. In *bouillon* which is kept undisturbed a characteristic appearance is produced, the culture medium remaining clear while a pellicle forms on the surface from which projections sprout downward (stalactite formation) toward a granular or grumous deposit which forms on the walls and on the bottom of the tube. In bouillon and most fluid media the growth is in the form of short or medium chains of very short, oval bacilli, which look almost like streptococci. It does not coagulate milk, but produces a slight acidity. It produces no indol in peptone media.

**Pathogenicity.**—Plague is a rodent disease transmissible to man. The most important rodent is the rat. Mice may become infected. Other rodents may be infected and be the means of maintaining the disease in endemic areas, as the marmot in Thibet or the ground squirrel in California. The tarbagan was primary source of infection in the epidemic of pneumonic plague in Manchuria (1910–11). Other species of rodents may later be found to be reservoirs of the disease.

These animals as well as guinea-pigs are easily infected artificially by feeding or application of the bacilli to the mucous membranes or to the skin. In the last, infection is sure to follow if a slight puncture or scratch is present. Monkeys and rabbits can also be infected. The bacilli may lose their virulence after prolonged artificial cultivation.

In *rodents* the disease may be either acute or chronic. A septicemia develops as a terminal event and fleas feeding at this time become infected. In *man,* the disease is bubonic, pneumonic or septicemic in type. Septicemia is the usual mode of termination of the bubonic and pneumonic types of the disease.

The diagnosis of natural infection in rats is made macroscopically, although occasionally the disease does occur without evident lesions. In acute plague the engorgement of the subcutaneous bloodvessels and the diffuse pink color of the subcutaneous tissues and muscles is strongly diagnostic. The superficial lymph nodes are very much enlarged and frequently surrounded by edema or hemorrhagic areas. The spleen is very much enlarged and soft. The liver is mottled with small hemorrhages and yellowish punctate areas of necrosis. Generally, there is an excess of fluid in the pleural cavity. In the more chronic disease, abscesses of the peripheral lymph nodes or more commonly mesenteric or splenic purulent or caseous foci are found. Frequently the rats submitted for examination are badly decomposed but pure cultures of the plague bacillus may be readily obtained by applying the material from the lesions to the freshly shaven abdomen of a guinea-pig. The plague bacilli penetrate the skin through the slight scarification due to dry shaving, whereas the other bacteria do not and a general infection results.

**Epidemiology.**—The disease is maintained in the rodents. Although direct contagion may occur, the most important mode of transfer is by rodent fleas. The bacilli taken up with infected blood multiply in the digestive tract of the flea but the mouth parts seem not to remain infected. Fleas may remain infected for weeks or even one or two months if the temperature is low. The mechanism of infection is probably the deposition of infected feces during the act of feeding or regurgitation of infected blood from a previous meal. Plague bacilli can invade the tissues through a flea bite, thus if a non-infected flea is allowed to bite a rat and a drop of plague cultures be placed on the bite infection results.

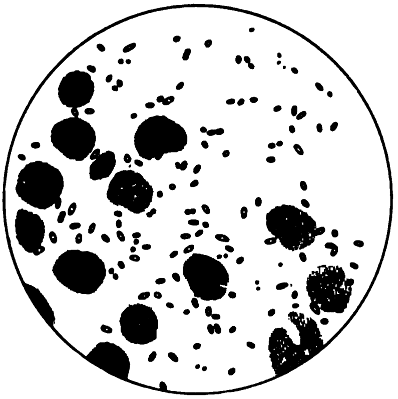

FIG. 155.—Bacilli in smear from acutely inflamed gland.

Bubonic plague in man is due to transfer of infection by means of fleas. The British plague commission proved conclusively that this is a fact. They allowed plague-infected rats to remain in contact with healthy rats in the absence of fleas and found that the healthy rat remained well, whereas, when fleas were placed in the cages the healthy rats became infected. They varied their experiments and corroborated their results. The pneumonic type may develop after infection by fleas due to blood invasion and pulmonary localization. Should this occur an outbreak of pneumonic plague may develop due to man-to-man contagion. This is most likely to occur during cold weather and under conditions of close contact. This form of disease is extremely contagious, with a mortality of 90 per cent. or more. The mortality of bubonic plague varies from 30 to 90 per cent.

Other biting insects than fleas may possibly be a factor in infection, for example bed-bugs. It is possible that human vermin were a factor in the widespread outbreaks of "black death" during the middle ages.

**Immunity.**—Like typhoid infection, a single attack of the plague bacillus protects, with rare exception, from a second infection. Yersin, Calmette, and Borrel have succeeded in immunizing animals against the bacillus of bubonic plague by the intravenous or intraperitoneal injection of dead cultures, or by repeated subcutaneous inoculation. They also succeeded in immunizing rabbits and horses, so that the serum afforded protection to small animals, after subcutaneous injection, of virulent cultures, and even cured those which had been inoculated, if administered within twelve hours after injection. The serum has considerable antitoxic as well as bactericidal properties. It also contains specific agglutinins which may be made use of in diagnosis. For use of vaccine and serum see Part III.

**Duration of Life Outside of the Body.**—In cultures protected from the air and light the plague bacilli may live ten years or more (Wilson). In the bodies of dead rats they may live for two months. In sputum from pneumonic cases the bacilli lived ten days. Upon sugar sacks, food, etc., they may live six to fifteen days.

**Resistance to Deleterious Influences.**—The bacilli resemble the colon bacilli in their reaction to heat and disinfectants.

**Bacteriological Diagnosis.**—Material is obtained in bubonic plague by puncture or incision of a lymph node, in pneumonic plague the sputum is employed. Direct smears if plague-like bacilli are present are valuable in making a rapid presumptive diagnosis, cultures and cutaneous inoculation of guinea-pigs should also be made. Antemortem blood cultures are frequently positive especially late in the disease. In postmortem examinations the heart blood and spleen should be examined. Wherever the material is badly contaminated or even decomposed the cutaneous method of inoculation of guinea-pigs should be resorted to. For rodents, see p. 468.

**Precipitin Reaction with Tissue Extracts.**—Warner,[1] following the technic of Ascoli as applied to anthrax lesions, has found this method of considerable value. The method is of great advantage in making a rapid presumptive diagnosis and is also of value in old or decomposed lesions which are not adapted for bacteriological examination. Here again it must not be forgotten that the reaction is only quantitatively specific, and must be adequately controlled. A negative reaction is not conclusive.

**Plague-like Disease in Rodents.**—McCoy and Chapin (1912) found an organism (Bacillus tularense) in a disease of Californian ground squirrels, which show lesions similar to those of plague. The bacilli have been cultivated by McCoy and Chapin on an egg-yolk medium. Wherry and Lamb[2] report two cases of conjunctivitis and lymphadenitis in man due to this bacillus, as well as an epidemic among wild rabbits.

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[1] Jour. Hygiene, 1914, xiv, 360.

[2] Jour. Am. Med. Assn., 1914, lxiii, 2041.

# CHAPTER XXXII.

## THE ANTHRAX BACILLUS

Anthrax is an acute infectious disease which is very prevalent among animals, particularly sheep and cattle. Geographically and zoölogically it is the most widespread of all infectious disorders. It is much more common in Europe and in Asia than in America. The ravages among herds of cattle in Russia and Siberia and among sheep in certain parts of France, Hungary, Germany, Persia, and India are not equalled by any other animal plague. Local epidemics have occasionally occurred in England, where it is known as splenic fever. In this country the disease in the lower animals is rare. In infected districts the greatest losses are incurred during the hot months of summer.

The disease also occurs in man as the result of infection either through the skin, the intestines, or in rare instances through the lungs. It is found in persons whose occupations bring them into contact with animals or animal products, as stablemen, shepherds, tanners, butchers, and those who work in wool and hair. During the recent war the increased importation of skins, hides and bristles from foreign countries resulted in a much larger number of cases of anthrax. New shaving brushes have been frequently found abundantly infected with anthrax spores and many cases of infection from them have been reported. Efficient measures for the disinfection of suspected hides, bristles, etc., would largely prevent infections from this organism. Two forms of the disease have been described—the external anthrax, or malignant pustule, and the internal anthrax, of which there are intestinal and pulmonary forms, the latter being known as "woolsorters' disease."

Owing to the fact that anthrax was the first infectious disease which was shown to be caused by a specific microörganism, and to the close study which it received in consequence, this disease has probably contributed more to our general knowledge of bacteriology than any other infectious malady.

Davaine, in 1863, announced to the French Academy of Sciences the results of his inoculation experiments, and asserted the etiological relations of the microörganism to the disease, with which his investigation showed it to be constantly associated. Pollender corroborated this statement. In 1877 Koch, Pasteur, and others established its truth by obtaining the bacillus in pure cultures, and showing that the inoculation of these cultures produced anthrax in susceptible animals as certainly as did the blood of an animal recently dead from the disease.

**Morphology.**—Slender, cylindrical, non-motile rods, having a breadth of $1\mu$ to $1.25\mu$, and ranging from $2\mu$ or $3\mu$ to $20\mu$ or $25\mu$ in length. Sometimes short, isolated rods are seen, and, again, shorter or longer chains or threads made up of several rods joined end to end. In suitable culture media very long, flexible filaments may be observed, which are frequently united in twisted or plaited cord-like bundles. (See Figs. 156 and 157.) These filaments in hanging-drop cultures, before the development of spores, appear to be homogeneous or nearly so; but in stained preparations they are seen to be composed of a series of rectangular, deeply stained segments. When obtained directly from the blood of an infected animal the free ends of the rods are slightly rounded, but those coming in contact with one another are often quite square. In cultures the ends may be a trifle thicker than the body of the cell and somewhat concave, giving the appearance of joints of bamboo. At one time much stress was laid upon these peculiarities as distinguishing marks of the anthrax bacillus; but it has been found that they are the effects of artificial cultivation and not necessarily characteristic of the organism under all conditions. The bacillus is inclosed in a transparent envelope or capsule, which in stained preparations (from albuminous material) may be distinguished by its taking on a lighter stain than the deeply stained rods which it surrounds.

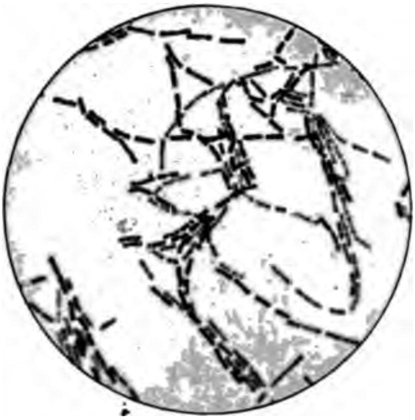

FIG. 156.—Anthrax bacillus. × 900 diameters. Agar culture.

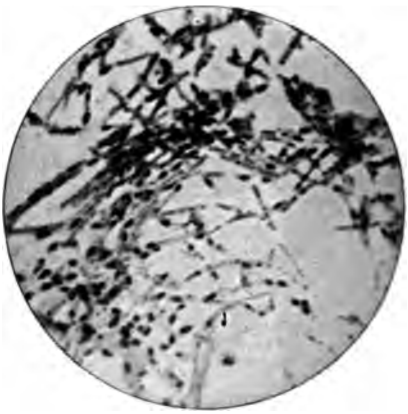

FIG. 157.—Spores heavily stained (in specimen red). Bodies of disintegrating bacilli faintly stained (in specimen blue). × 1000 diameters.

**Spore Formation.**—In the presence of free oxygen in cultures spores are developed in the bacilli. These spores are elliptic in shape and about one and a half times longer than broad. They first appear as small, refractive granules distributed at regular intervals, one in each rod. As the spore develops the mother-cell becomes less and less distinct, until it disappears altogether, the complete oval spore being set free by its dissolution. (See Fig. 157 and Plate III, Fig. 22). Irregular sporulation sometimes takes place, and occasionally there is no spore formation as in varieties of non-sporebearing anthrax.

Sporeless varieties have been produced artificially by cultivating the typical anthrax bacillus under certain conditions, among which may be mentioned the addition of antiseptics, as carbolic acid, and of continued high temperature (43° C.). Varieties differing in their pathogenic power may also be produced artificially. Pasteur produced an "attenuated virus" by keeping his cultures for a considerable time before replanting them upon fresh soil.

Anthrax cultures containing spores retain their vitality for years; in the absence of spores the vitality is much more rapidly lost. When grown in liquids rich in albumin the bacilli attain a considerable degree of resistance; thus dried anthrax blood has been found to retain its virulence for sixty days, while dried bouillon cultures only did so for twenty-one days. Dried anthrax spores may be preserved for many years without losing their vitality or virulence. They also resist a comparatively high temperature. Exposed in dry air they require a temperature of 140° C. maintained for three hours to destroy them; but suspended in a liquid they are destroyed in four minutes by a temperature of 100° C.

**Staining.**—The anthrax bacillus *stains* readily with all the anilin colors, and also by Gram's method, when not left too long in the decolorizing solution. In sections good results may be obtained by the employment of Gram's solution in combination with carmine, but when only a few bacilli are present this method is not always reliable, as some of the bacilli are generally decolorized.

**McFadyean-Heine Methylene-blue Reaction.**—In imperfectly fixed film preparations (pass through flame three times in a second with film side up) the capsule disintegrates. When a solid film is stained for a few seconds in an old solution of methylene blue, washed in water and dried with filter paper, the bacteria are surrounded by a varying amount of a reddish-purple amorphous or fixed granular deposit. McFadyean says this does not occur with other morphologically similar bacteria.

**Biology.**—The anthrax bacillus grows easily in a variety of nutrient media at a temperature from 18° to 43° C., 37° C., being the most favorable temperature. Under 12° C. no development takes place as a rule, though by gradually accustoming the bacillus to a lower temperature it may be induced to grow under these conditions. Under 14° C. and above 43° C. spore formation ceases. The lower limit of growth and of sporulation is of practical significance in determining the question whether development can occur in the bodies of animals dead from anthrax when buried at certain depths in the earth. Kitasato has shown that at a depth 1.5 meters the earth in July has a temperature of 15° C. at most, and that under these conditions a scanty sporulation of anthrax bacilli is possible, but that at a depth of 2 meters sporulation no longer occurs. The anthrax bacillus is aërobic—that is, its growth is considerably enhanced by the presence of oxygen—but it grows also under anaërobic conditions, as is shown by its growth at the bottom of the line of puncture in stick cultures in solid media; but under these conditions it no longer produces the peptonizing ferment which it does with free access of air. Furthermore, the presence of free oxygen is absolutely necessary for the formation of spores, while carbonic acid gas retards sporulation. This explains, perhaps, why sporulation does not take place within the animal body either before or after death.

It is also capable of leading a saprophytic existence. The bacillus is non-motile.

**Growth in Gelatin.**—In *gelatin-plate cultures*, at the end of twenty-four to thirty-six hours at 24° C., small, white, opaque colonies are developed, which, under a low-power lens, are seen to be dark gray in the center and surrounded by a grayish, irregular border, made up of wavy filaments. As the colony develops on the surface of the gelatin these wavy filaments spread out, until finally the entire colony consists of a light gray, tangled mass, which has been likened to a Medusa head (Fig. 158).

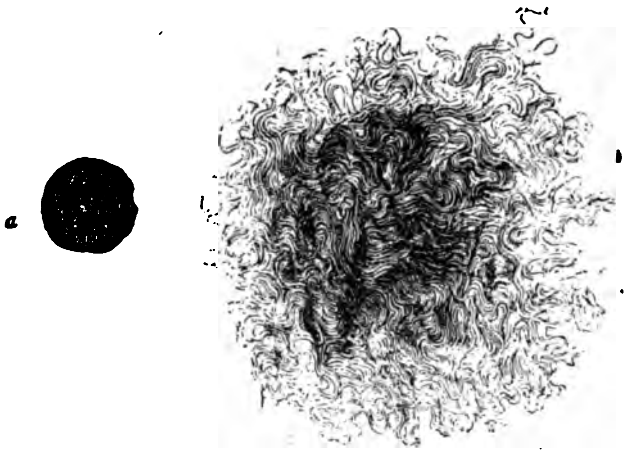

FIG 158.—Colonies of Bacillus anthracis upon gelatin plates: *a*, at the end of twenty-four hours; *b*, at the end of forty-eight hours. × 80. (F. Flügge.)

At the same time the gelatin begins to liquefy, and the colony is slowly surrounded by the liquefied medium, upon the surface of which it floats as an irregular, white pellicle. In *gelatin-stick cultures* at first, development occurs along the line of puncture as a delicate white thread, from which irregular, hair-like projections soon extend perpendicularly into the culture medium, the growth being most luxuriant near the surface, but continuing also below. At the end of two or three days liquefaction of the medium commences at the surface and gradually progresses downward.

**Growth on Agar.**—The growth on *agar-plate cultures* in the incubator at 37° C. is similar to that on gelatin, and is still more characteristic and beautiful in appearance. A grayish-white layer is formed on the surface within twenty-four hours, which spreads rapidly and is seen to be made up of interlaced threads.

**Growth in Milk.**—A small amount of acidity is produced. The milk is curdled and then digested by a rennin-like ferment.

**Growth in Bouillon.**—The growth is characterized by the formation of flaky masses, which sink as a sediment to the bottom of the tube, leaving the supernatant liquid clear.

**Pathogenesis.**—The anthrax bacillus is pathogenic for cattle, sheep (except the Algerian race), horses, swine, mice, guinea-pigs, and rabbits. Rats, cats, dogs, chickens, owls, pigeons, and frogs are but little susceptible to infection. Small birds—the sparrow particularly—are somewhat susceptible. Man, though subject to local infection and occasionally to internal forms of the disease, is not as susceptible as some of the lower animals.

In the more susceptible animals the anthrax bacillus produces a true septicemia. Among test animals mice are the most susceptible, succumbing to very minute injections of a slightly virulent virus; next guinea-pigs, and then rabbits, both of these animals dying after inoculation with virulent bacilli. Infection is most promptly produced by introduction of the bacilli into the circulation or the tissues, but inoculation by contact with wounds on the skin also causes infection. It is difficult to produce infection by the ingestion even of spores; but it may readily be caused by inhalation, particularly of spores.

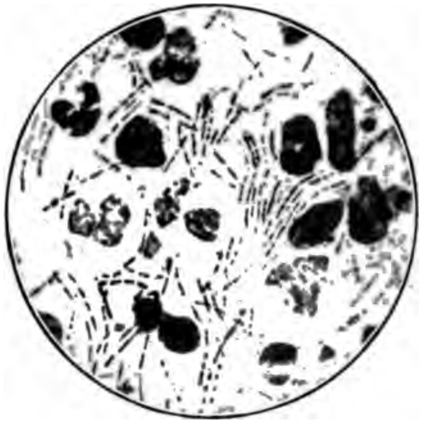

Fig. 159.—Section of liver from mouse dead of anthrax septicemia. × 1000 diameters. (From Itzerott and Niemann.)

Subcutaneous injections of these susceptible animals results in death in from one to three days. Comparatively little local reaction occurs immediately at the point of inoculation, but beyond this there is an extensive edema of the tissues. Very few bacilli are found in the blood in the larger vessels, but in the internal organs, and especially in the capillaries of the liver, the kidneys and the lungs, they are present in great numbers. In some places, as in the glomeruli of the kidneys, the capillaries will be seen to be stuffed full of bacilli, and hemorrhages, probably due to rupture of capillaries by the mechanical pressure of the bacilli which are developing within them, may occur. The pathological lesions in animals infected by anthrax are not marked except in the spleen, which, as in other forms of septicemia, is greatly enlarged.

**Occurrence in Cattle and Sheep.**—Cattle and sheep are affected chiefly with the intestinal form of anthrax, infection in these animals commonly resulting from the ingestion of food containing spores. The bacillus itself, in the absence of spores, is quickly destroyed by the gastric juice. The disease usually takes a rapid course, and the mortlaity is high—70 to 80 per cent. The pathological lesions consist of numerous ecchymoses, enlargement of the lymphatic glands, serous, fatty, and hemorrhagic infiltration of the mediastinum and mesentery, of the mucous membranes of the pharynx and larynx, and particularly of the duodenum, great enlargement of the spleen, and parenchymatous changes in the lymphatic organs. The blood is very dark and tar-like. Bacilli are present, especially in the lymph spaces, in enormous masses.

Sheep are also subject to external anthrax, infection taking place by way of the skin; cattle are seldom infected in this way. At the point of inoculation there develops a hard, circumscribed boil—the so-called anthrax carbuncle; or there may be diffuse edema with great swelling of the parts. When death occurs the appearances are similar to those in intestinal anthrax, except that the duodenum is usually less affected; but in all cases metastasis occurs in various parts of the body, brought about, no doubt, by previous hemorrhages.

**Occurrence in Man.**—The disease does not occur spontaneously in man, but always results from infection, either through the skin, the intestines, or occasionally by inhalation through the lungs. It is usually produced by cutaneous infection through inoculation of exposed surfaces—the hands, arms, or face. Infection of the face or neck would seem to be the most dangerous, the mortality in such cases being 26 per cent., while infection of the extremities is rarely fatal.

External anthrax in man is similar to this form of the disease in animals. There are two forms: malignant pustule or carbuncle, and, less commonly, malignant anthrax edema.

*In malignant pustule,* at the site of inoculations, a small papule develops, which becomes vesicular. Inflammatory induration extends around this, and within thirty-six hours there is a dark, brownish eschar in the centre, at a little distance from which there may be a series of small vesicles. The brawny induration may be extreme. There may also be considerable edema of the parts. In most cases there is no fever; or the temperature at first rises rapidly and the febrile phenomena are marked. Death may take place in from three to five days. In cases which recover the symptoms are slighter. In the mildest form there may be only slight swelling.

*Malignant anthrax edema* occurs in the eyelids, and also in the head and neck, sometimes the hand and arm. It is characterized by the absence of the papule and vesicle forms, and by the most extensive edema. The edema may become so intense that gangrene results; such cases usually prove fatal.

The bacilli are found on microscopic examination of the fluid from the pustule shortly after infection; later the typical anthrax bacilli are often replaced by involution forms. In this case resort may be had to cultures, animal inoculation, or examination of sections of the extirpated tumor. The bacilli are not present in the blood until just before death. Along with the anthrax bacilli pyogenic cocci are often found in the pustule penetrating into the dead tissue.

*Internal anthrax* is much less common in man; it does, however, occur now and then. There are two forms of this: the intestinal form, or mycosis intestinalis, and the pulmonic form, or wool-sorters' disease.

*Intestinal anthrax* is caused by infection thrugh the stomach and intestines, and results probably from the eating of raw flesh or unboiled milk of diseased animals. That the eating of flesh from infected animals is comparatively harmless is shown by Gerlief, who states that of 400 persons who were known to have eaten such meat not one was affected with anthrax. On the other hand, an epidemic of anthrax was produced among wild animals, according to Jansen, by feeding them on infected horse flesh. It is evident, therefore, that there is a possibility of infection being caused in this way. The recorded cases of intestinal anthrax in man have occurred in persons who were in the habit of handling hides, hair, etc., which were contaminated with spores; in those who were conducting laboratory experiments, and rarely it has been produced by the ingestion of food, such as raw ham and milk. The symptoms produced in this disease are those of intense poisoning, chill, followed by vomiting, diarrhea, moderate fever, and pains in the legs and back. The pathological lesions are similar to those described in animals.

*Wool-sorters' disease,* or pulmonic anthrax, is found in large establishments in which wool and hair are sorted and cleansed, and caused by the inhalation of dust contaminated with anthrax spores. The attack comes on with chills, prostration, then fever. The breathing is rapid, and the patient complains of pain in the chest. There may be a cough and signs of bronchitis. The bronchial symptoms in some instances are pronounced. Death may occur in from two to seven days. The pathological changes produced are swelling of the glands of the neck, the formation of foci of necrosis in the air passages, edema of the lungs, pleurisy, bronchitis, enlargement of the spleen, and parenchymatous degenerations. Meningitis has also been reported.

**Immunity against Anthrax Infection.**—The question of the cause of the natural immunity in the dog and some other animals has been much studied. Phagocytosis is supposed to be the chief agent in these cases. In susceptible animals immunity may be produced artificially. The efforts of Pasteur to effect immunity in animals by preventive inoculations of "attentuated virus" of the anthrax bacillus opened a new field of productive original research. Following in his wake many others have devised methods of immunization against anthrax infection; but the one adopted by Pasteur, Chamberland, and Roux has alone been practically employed on a large scale. Anthrax strains of two degrees of virulence, attenuated by growing at 42° C., are used for inoculation.

**Serum Treatment.**—This is discussed in Part III.

**Bacterial Cultures for Diagnosis.**—The detection of the anthrax bacillus is ordinarily not difficult, as this organism presents morphological, biological, and pathogenic characteristics which distinguish it from all other bacteria. In the later stages of the disease, however, the bacilli may be absent or difficult to find, and cultivation on artificial media and experimental inoculation in animals are not always followed by positive results. Even in sections taken from the extirpated pustule it is sometimes difficult to detect the bacilli. In such cases only a probable diagnosis of anthrax can be made. It should be remembered that the bacilli are not often found in the blood in large numbers until shortly before death, and then only in varying quantity; thus blood examinations often give negative results, though the bacilli may be present in large numbers in the spleen, kidneys, and other organs of the body. The suspected material should be streaked over nutrient agar plates and inoculated into melted agar for pour plates. It should also be inoculated in mice and rabbits. Direct smears should also be made. Such freshly opened lesions contain no spores so the material cannot be heated to get rid of contaminants.

**Isolation from Hair or Bristles.**—Here we may take advantage of the fact that the spores are present. The suspected material is rubbed up with salt solution in a mortar. Half of the emulsion is subjected to heat to kill vegetative contaminants. Both portions are then centrifuged and from the precipitates a number of dilutions in pour-agar plates are made from which typical colonies are fished in from twenty-four to forty-eight hours and tested culturally and by animal inoculations. Mice and rabbits are also inoculated from the precipitates.

**Differential Diagnosis.**—Among other bacteria which may possibly be mistaken for anthrax bacilli are *Bacillus subtilis* and the bacillus of malignant edema. The former is distinguished by its motility, by various cultural peculiarities, and by being non-pathogenic. The latter differs from the anthrax bacillus in form and motility, in being decolorized by Gram's solution, in being a strict anaerobe, and in various pathogenic properties. (See also description in chapter on Water Analysis.)

The diagnosis of internal anthrax in man is by no means easy, unless the history points definitely to infection in the occupation of the individual. In cases of doubt, cultures should be made and inoculations performed in animals. Rabbits are more specificially susceptible than mice.

**Precipitin Reaction.**—Ascoli,[1] taking advantage of the known fact that bacterial precipitinogen is resistant to heat developed a diagnostic procedure of considerable value. An immune anthrax serum is employed of sufficient titre to give rapid precipitation but it should not have an unusual content of common antibodies, or confusing reactions will be obtained with other than from the anthrax bacillus. That is, the reaction is not completely but only quantitatively specific. The suspicious lesion is broken up and boiled with 5 to 10 parts of saline, the extract filtered through paper or cleared by centrifuge. In place of salt solution, a dilution of 1 to 1000 acetic acid may be employed. In making extracts from agar cultures we have found that suspending and extracting the growths in 5 c.c. of salt solution gives satisfactory results. The extract is superimposed on the serum to elicit a ring reaction. With experience and knowledge of the degree of specificity of the serum employed very reliable results are obtainable. The method is of especial value in *inspection control of meat.* Old or decomposed lesions will still give a reaction. Naturally a negative result with tissue extracts has not the same value as a positive reaction. The number of bacilli and the amount of bacillary products in the tissue must be considerable before a definitely positive reaction can be obtained.

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[1] Zeitsch. f. Immunforsch., 1911, xi, 103.

# CHAPTER XXXIII.

# ANAËROBIC BACILLI.

In this chapter we are grouping several species of bacteria which have in common only the characteristic that they cannot grow in pure cultures in the presence of free oxygen. Those of most interest to us are B. tetani, B. (anthracis) symptomatici, B. (edematis) maligni, B. welchii and related forms, B. botulinus and B. fusiformis.

## THE BACILLUS AND THE BACTERIOLOGY OF TETANUS.

Tetanus is a disease which is characterized by a gradual onset of general spasms of the voluntary muscles, commencing in both man and the horse most often in the muscles of the jaw and neck, and extending in severe cases to the muscles of the body. The disease is usually associated with a wound received from four to fourteen days previously.

Tetanus has been reported for many centuries. The writings of Hippocrates clearly describe the symptoms. In 1884 Nicolaier, under Flügge's direction, produced tetanus in mice and rabbits by the subcutaneous inoculation of particles of garden earth. The Italians, Carle and Rattone, had just before demonstrated that the pus of an infected wound from a person attacked with tetanus could produce the same disease in rabbits. Finally, Kitasato, in 1889, obtained the bacillus for tetanus in pure culture and described his method of obtaining it and its biological characters.

**Occurrence in Soil, etc.**—The tetanus bacillus occurs widely throughout the world as a common inhabitant of the soil, especially in places where manure has been thrown, being abundant in many localities not only in the superficial layers, but also at the depth of several feet. It has been found in many different substances and places—in hay dust, in horse and cow manure (its normal habitat is the intestine of the herbivora), in the mortar of old masonry, in the dust from horses' hair, and in the dust in rooms of houses, barracks, and hospitals.

The tetanus bacilli are more numerous in certain localities than in others—for example, some parts of Long Island and New Jersey have become notorious for the number of cases of tetanus caused by small wounds—and they are fairly common in New York City. As a rule they are more abundant in regions where the temperature is high. In some islands and countries in the tropics cases of puerperal tetanus and tetanus in the newborn are very frequent. Tetanus bacilli are found in the intestines of about 15 per cent. of horses and calves living in the vicinity of New York City. They are also present to a somewhat less extent in the intestines of other animals and of man.

**Morphology.**—From young gelatin cultures the bacilli appear as motile, slender rods, with rounded ends, 0.3μ t 0.5μ in diameter by 2μ to 4μ in length, usually occurring singly, but, especially in old cultures, often growing in long threads. They form round or nearly round spores, thicker than the cell (from 1μ to 1.5μ in diameter), occupying one of its extremities and giving to the rods the appearance of small pins (Fig. 160.)

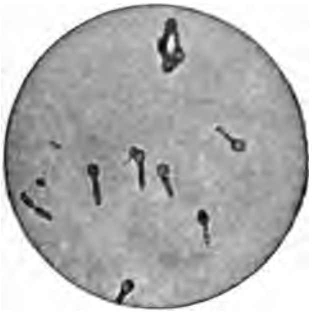

FIG. 160.—Tetanus bacilli with spores distending ends. × 1100 diameters.

**Staining.**—The bacillus is *stained* with the ordinary anilin dyes, and is not decolorized by Gram's method. The spores are readily stained and may be demonstrated by double-staining with Ziehl's method. The flagella are fairly easily stained on freshly developed bacilli taken from cultures which have been at short intervals several times transplanted.

**Biology.** — An *anaërobic, liquefying, moderately motile* bacillus. It has abundant peritrichic flagella. During the period of spore formation it is not motile. It grows slowly at temperatures from 20° to 24°C., and best at 38° C., when, within twenty-four to thirty hours it forms spores. At temperatures of 20° to 24° C., spores form in from six to ten days. It will not grow in pure culture in the presence of oxygen, but grows well in an atmosphere of hydrogen gas. If planted with certain other bacteria the tetanus bacillus grows luxuriantly in the presence of oxygen.

**Growth in Media.**—The bacillus of tetanus grows anaërobically in nutrient gelatin and agar of a slightly alkaline reaction. The addition to the media of 1.5 per cent. of glucose causes the development to be more rapid and abundant. It also grows abundantly in alkaline bouillon in an atmosphere of hydrogen. On *gelatin plates* the colonies develop slowly; they resemble somewhat the colonies of the *Bacillus subtilis*, and have a dense, opaque center surrounded by fine, diverging rays. Liquefaction takes place more slowly, however, than with Bacillus subtilis, and the resemblance to these colonies is soon lost.

The colonies on *agar* are quite characteristic. To the naked eye they present the appearance of light, fleecy clouds; under the microscope, a tangle of fine threads.

The *stab cultures in gelatin* exhibit the appearance of a cloudy, linear mass, with prolongations radiating into the gelatin from all sides (arborescent growth). Liquefaction takes place slowly, generally with the production of gas. In *stab cultures in agar* a growth occurs presenting the appearance of a miniature pine tree. *Alkaline bouillon* is rendered somewhat turbid by the growth of the tetanus bacillus. In all cases a production of gas results, accompanied by a characteristic and very disagreeable odor. It develops in *milk* without causing coagulation.

**Resistance of Spores to Deleterious Influences.**—The spores of the tetanus bacillus are very resistant to outside influences; in a desiccated condition they may retain their vitality for several years, and are not destroyed in two and a half months when present in putrefying material. They withstand an exposure of one hour at 80° C., but are usually killed by an exposure of ten minutes at 105° C. to live steam. They resist the action of 5 per cent. carbolic acid for ten hours. A 5 per cent. solution of carbolic acid, however, to which 0.5 per cent. of hydrochloric acid has been added, destroys them in two hours. They are killed when acted upon for three hours by bichloride of mercury (1 to 1000), and in thirty minutes when 0.5 per cent. HCl is added to the solution. Silver nitrate solutions destroy the spores of average resistance in one minute in 1 per cent. solution and in about five minutes in 1 to 1000 solution.

With regard to the persistence of tetanus spores upon objects where they have found a resting place, Henrijean reports that by means of a splinter of wood which had once caused tetanus he was able after eleven years again to cause the disease by inoculating an animal by means of the same splinter.

**Isolation of Pure Culture.**—The growth of the tetanus bacillus in the animal body is comparatively scanty, and is usually associated with that of other bacteria; hence the organism is difficult to obtain in pure culture. The method of procedure which is most successful, consists in inoculating the tetanus-bearing material (pus or tissue from the inoculation wound) into tubes of freshly sterilized slightly alkaline nutrient agar or fermentation tubes of glucose bouillon to each of which a piece of fresh sterile tissue has been added, and incubating at 37° C. After the tetanus spores have formed as shown in microscopic preparations from the sediment about the piece of tissue, heat for one-half hour at 80° C. to destroy the associated bacteria and subinoculate in broth and make plates. If the tetanus bacilli are the only spore-bearing bacteria present, pure cultures are readily obtained; when other spore-bearing anaërobic bacteria are present, the isolation of a pure culture may be a matter of difficulty, but even then the presence of tetanus toxin in the culture fluid, shown by the inoculation of animals, will indicate the presence of tetanus bacilli.

**Pathogenesis.**—In mice, guinea-pigs, rabbits, horses, cattle, goats and a number of other animals inoculations of pure cultures of the tetanus bacillus cause tetanus after an incubation of from one to four days. In the smaller animals tetanus usually develops first in the muscles nearest the point of inoculation. A mere trace of an old culture—only as much as remains clinging to a platinum needle—is often sufficient to kill very susceptible animals like mice and guinea-pigs. Other animals require a larger amount. Rats and birds are but little susceptible, and fowls scarcely at all. These never develop tetanus from natural infection. It is a remarkable fact that an amount of toxin sufficient to kill a hen would suffice to kill 500 horses. It is estimated that if 1 gram of horse requires 1 part of toxin to kill, then 1 gram of guinea-pig requires 6 parts, 1 of mouse 12, of goat 24, of dog 500, of rabbit 1500, of cat 6000, of hen 360,000. Horses frequently develop tetanus after injuries or operations. Cultures from different cases vary in their toxicity. On the inoculation of less than a fatal dose in test animals a local tetanus may be produced, which lasts for days and weeks and then ends in recovery. On killing the animal there is found at autopsy, just at the point of inoculation, a hemorrhagic spot, and no changes other than these here or in the internal organs. A few tetanus bacilli may be detected locally with great difficulty, often none at all; possibly a few may be found in the region of the neighboring lymphatic glands and even in the blood. From this scanty occurrence of bacilli the conclusion has been reached that the bacilli of tetanus, when inoculated in pure culture, do not multiply to any great extent in the living body, but only produce lesions through the absorption of the poison which they develop at the point of infection. It has been found that pure cultures of tetanus, after the germs have sporulated and the toxins been destroyed by heat, can be injected into animals without producing tetanus. But if pathogenic streptococci or staphylococci or even non-pathogenic organisms are injected simultaneously with the spores, or if there is an effusion of blood at the point of injection, or if there was a previous or later bruising of the tissues, the animals surely die of tetanus.

**Natural Infection.**—Here the infection may be considered as probably produced by the bacilli in their spore state, and the conditions favoring infection are almost always present. A wound of some kind has occurred, penetrating at least through the skin, though perhaps of a most trivial character, such as might be caused by a dirty splinter of wood, and the bacilli or their spores are thus introduced from the soil in which they are so widely distributed. If in any given case, the tissues being healthy, the ordinary saprophytic germs are killed by proper disinfection at once, a mixed infection does not take place, and tetanus will not develop. If, however, other bacteria, especially pathogenic or putrefactive types accompany the tetanus bacilli, or if the tissues infected be bruised or lacerated, the spores may develop and produce the disease. Gelatin and catgut are occasionally found to contain tetanus spores.

**Tetanus in Man.**—Man and almost all domestic animals are subject to tetanus. It is a comparatively rare disease in the United States, during times of peace, except after the Fourth of July celebration, when a considerable number of cases develop. Injuries causing crushing of tissues with accompanying contamination are the most likely to be followed by tetanus. Until immunization became the practice more than one hundred persons yearly developed tetanus after blank cartridge wounds. On examination of an infected individual very little local evidence of the disease can be discovered. Generally at the point of infection, if there is an external wound, some pus is to be seen, in which, along with numerous other bacteria, tetanus bacilli or their spores may be found. Although rather deep wounds are usually the seat of infection, at times such superficial wounds as an acne pustule or a vaccination may give the occasion for infection. In rare cases tetanus has developed from the infection of necrotic mucous membranes as in diphtheria. Not only undoubted traumatic tetanus, but also all the other forms of tetanus, are now conceded to be produced by the tetanus bacillus—puerperal tetanus, tetanus neonatorum, and idiopathic tetanus. In tetanus neonatorum, infection is introduced through the navel, in puerperal tetanus through the inner surface of the uterus. It should be borne in mind that when there is no external and visible wound there may be an internal one. The lesions in the nervous system are still obscure. Congestion, cellular exudate into the perivascular spaces, and chromatolysis of the ganglion cells are common.

**Toxins of the Tetanus Bacillus.**—It is evident from the localization of the tetanus bacilli almost wholly at the point of inoculation and their moderate multiplication at this point that they exert their action through the production of powerful toxins. These toxins are named, according to their action, the tetanospasmin and the tetanolysin. The first only is of importance. One ten-thousandth of a cubic centimeter of the filtrate of an eight-day bouillon culture of a fully virulent bacillus is sufficient to kill a mouse. The purified and dried tetanus toxin prepared by Brieger and Cohn was surely fatal to a 15-gram mouse in a dose of 0.000005 gram. The toxin is precipitated by saturating the broth with ammonium sulphate and having been collected on the filter is compressed to eliminate the fluid clinging to it. The appalling strength of tetanus toxin may readily be appreciated when it is stated that it is twenty times as poisonous as dried cobra venom.

The quantity of the toxin produced in nutrient media varies according to the age of the culture, the composition of the culture fluid, reaction, completeness of the exclusion of oxygen, etc. For some reason more toxin develops in broth inoculated with masses of tetanus spores in nutrient agar, than with bacilli. The variation in strength is partly due to the extreme sensitiveness of the toxin, which deteriorates on keeping at blood heat or on exposure to light. It is sensibly affected by most chemical reagents and is largely destroyed by heating to 55° to 60° C. for a few minutes. It retains its strength best when protected from heat, light, oxygen, and moisture.

The tetanus cultures retain their ability to produce toxins unaltered when kept under suitable conditions; but when subjected to deleterious influences they may entirely lose it.

**Production of Toxin for Immunization of Horses.**—Following is the method of handling stock cultures and of producing toxin, modified by Wilcox[1] in our laboratory from the method used by Anderson and Leake.[2]

*Stock Cultures.*—The stock cultures are grown on a semisolid agar medium neutral to phenolphthalein.

To transfer cultures, one of the semisolid agar cultures is melted and 1 c.c. is added to a freshly melted semisolid agar tube, at least ten subcultures being thus made from one stock culture. After inoculation, the tubes are cooled, the plugs immersed in paraffin and the tubes incubated at 37° C. After one week's incubation the cultures are stored in the ice-chest, where they may be kept for six months without affecting their ability to produce toxin.

*Preliminary Cultivation.*—Fill potato tubes with about 40 c.c. of the toxin broth (Chapter IV) sterilize for one and one-half hours on the first day and one hour on the second day. These tubes may be kept for two weeks, up to which time they will give satisfactory growth. To make the first transfer for the preliminary cultivation add 8 c.c. of the melted semisolid agar stock culture of the B. tetani to two tubes of glucose broth from which the air has been previously expelled by heating in the Arnold for fifteen to twenty minutes, and which have been cooled down to about 50° C. These tubes are incubated for twenty-four hours and the next day, two freshly heated tubes of broth are inoculated with

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[1] Jour. Bact., 1916, i, 333.

[2] Jour. Med. Res., 1915, xxxiii, 235.

5 c.c. of the glucose broth cultures planted the previous day. On the third day determine the number of flasks that are to be inoculated and inoculate as many freshly heated glucose broth tubes from the second glucose broth generation as there are flasks. Anderson calls for at least six or seven generations in the glucose broth before the inoculation of the toxin broth, but at the Research Laboratory it has been found that three generations, or even two if need be, are sufficient for obtaining a toxicity of 1 to 25,000.

*Inoculation of Toxin Broth.*—The flasks of toxin broth after the second sterilization in the Arnold are ready for inoculation. The broth may be cooled down to 55° to 60° C. by allowing the flasks to stand at room temperature, or in a more rapid way by placing the hot flasks in a large sink, to which cool, and then cold, water is added until the lower portions of the flasks are covered. When the bottoms of the flasks are cool to the hand, the portions above the water being still very hot, the inoculation may be made as follows:

The plugs are carefully removed, the necks flamed and the plugs replaced. In a similar way, the mouths of the culture tubes are sterilized and then, partly removing the plug of a flask the contents of a potato tube are poured rapidly into a flask. If one prefers, the broth culture may be transferred by using a pipette but the former method has been used here without subsequent contamination and found very satisfactory. After inoculation, the flasks are incubated for fifteen days at 36° to 37° C., care being taken to exclude all light from them.

The flasks at the end of twenty-four to forty-eight hours show a diffuse cloudiness with the formation of gas bubbles on the surface of the broth. Toward the end of two weeks the gas bubbles usually disappear, while the cloudiness persists and a light precipitate forms at the bottom of the flasks. If it is not convenient to filter on the fifteenth day, the cultures may be kept in the incubator until the twentieth day without a loss of toxicity, but from the twentieth day to the twenty-fifth day the toxin loses about 20 per cent. in potency.

*Filtration.*—All glassware, filters, etc., should be neutral to phenolphthalein and the greatest care should be taken to exclude light, either direct or indirect, by darkening the room and by covering the filtering apparatus with dark cloths, ordinary black cambric being used.

The broth cultures are first passed through Buchner filters about eight inches in diameter, which have been packed with a layer of finely shredded paper pulp 0.25 inch in thickness. It is of importance that the pulp should be so well packed that the filtrate is absolutely clear, otherwise it will clog the Berkefeld filter. The first filtrate, about 200 c.c., which passes through the Buchner is discarded, as it contains a considerable amount of water from the pulp, and then the filtering of the toxin may proceed. If, after passing 8 to 14 liters through the pulp, the filtrate begins to appear cloudy, the pulp must be discarded and the Buchner repacked. The clear filtrate is then passed through a sterile Berkefeld filter, and 10 per cent. of a 5 per cent. solution of carbolic acid solution is added to the toxin which is now placed in the ice-chest, ready for testing its potency.

*Potency Test.*—Two 350-gram guinea-pigs are inoculated subcutaneously over the abdomen with 1 c.c. of a dilution of 1 to 15,000 and 1 to 25,000 of the toxin respectively. If the toxin has a potency of 25,000 the pig receiving the 15,000 dilution will die on the second to third day and the pig receiving the 25,000 dilution should die on the fourth day. If both pigs die with symptoms of tetanus before the fourth day, the toxin is stronger than 25,000 and a higher dilution should be tested.

**Action of Tetanus Toxin in the Body.**—After the absorption of the poison there is a lapse of time before any effects are noticed. In experimental animals with an enormous amount, such as 90,000 fatal doses, this is about nine hours; with 30,000, ten; with 3000, twelve; with 300, twenty hours; with ten fatal doses, thirty-six to forty-eight hours; with two fatal doses, two or three days; with one fatal dose, four to seven days.

Less than a fatal dose will produce local symptoms. The parts first to be affected with tetanus in these animals are usually the muscles lying in the vicinity of the inoculation—for instance, the hindfoot of a mouse inoculated on that leg is first affected, then the tail, the other foot, the back and chest muscles on both sides, and the forelegs, until finally there is a general tetanus of the entire body. In mild cases, or when a dose too small to be fatal has been received, the tetanic spasm may remain confined to the muscles adjacent to the point of inoculation or infection. The symptoms following a fatal dose of toxin vary greatly with the method of injection. Intraperitoneal injection is followed by symptoms which can hardly be distinguished from those due to many other poisons. Injection into the brain is followed by restlessness and epileptiform convulsions. In man the first symptoms are usually those of a contraction of the muscles of the lower jaw and then those of the neck. In about one-third of the human cases, the muscles about the site of inoculation are first affected.

**Presence of Tetanus Toxin in the Blood.**—The blood during the first four days of the disease usually contains toxin. After that time antitoxin usually develops and soon makes the blood antitoxic. In St. Louis some years ago the serum of a horse dying of tetanus was given by accident in doses of 5 to 10 c.c. to a number of children, with the development of fatal tetanus. In this connection Bolton and Fisch showed by a series of experiments that much toxin might accumulate in the serum before symptoms became marked.

**Tetanus Antitoxin.**—Behring and Kitasato were the first to show the protective and curative effects of the blood serum of immunized animals. It was found that animals could be protected from tetanus infection by the previous or simultaneous injection of tetanus antitoxin, provided that such antitoxin serum was obtained from a thoroughly immunized animal. From this it was assumed that the same result could be produced in natural tetanus in man. Unfortunately, however, the conditions in the natural disease are very much less favorable, inasmuch as treatment is usually commenced not shortly after the infection has taken place, but some hours after the appearance of tetanic symptoms, when the poison has already attacked the cells of the central nervous system.

**The Production of Tetanus Antitoxin for Therapeutic Purposes.**—(See Chapter VII.)

**Preventive and Therapeutic Application of Tetanus Antitoxin.**—See Part III.

**Persistence of Antitoxin in the Blood.**—Ransom has clearly shown that the tetanus antitoxin, whether directly injected or whether produced in the body, is eliminated equally rapidly from the blood of an animal, provided that the serum was from an animal of the same species. If from a different species it is much more quickly eliminated.

The same author found some interesting facts in testing the antitoxic values of the serum of an immunized mare, of its foal, and of the milk. The foal's serum was one-third the strength of the mare's and one hundred and fifty times that of the mare's milk. In two months the mare's serum lost two-thirds in antitoxic strength, the foal's five-sixths, and the milk one-half.

**Theories as to the Methods by which the Toxin Produces its Effects.**—Gamprecht and Stintzing concluded from their experiments that the toxin from the wound passed to the central nervous system partly directly by the peri- and endoneural lymph spaces of the nerves which directly connected with subdural space and partly indirectly from the blood. The local tetanus they considered as due to the contact of the poison with the motor end-plates. The experiments of Meyer and Ransom and of Marie and Morax have proved to them that the poison is transported to the central nervous system by the way of the motor nerves—and by no other channel. These authors thought that they had shown that the essential element for the absorption and transportation of the toxin is not the lymph channels, but the axis-cylinder, the intramuscular endings of which the toxin penetrates. The poison is taken up quite rapidly. Marie and Morax were able to demonstrate the poison in the corresponding nerve trunk (sciatic) one and a half hours after the injection. Absorption, however, and conduction are dependent to a large extent on the nerves being intact. A nerve cut across takes very much longer to take up the poison (about twenty-four hours), and a degenerated nerve takes up no poison whatever. In other words, we see that section of the nerve prevents the absorption of the poison by way of the nerve channels. Similarly section of the spinal cord prevents the poison from ascending to the brain. The poison which passed through the general lymph channels to the blood was partly returned to the tissue fluids throughout the body and taken up by nerve endings and thus produced general tetanus.

According to Meyer and Ransom, the reason why the sensory nerves do not play any role in the conduction of the poison lies in the presence of the spinal ganglion, which places a bar to the advance of the poison. Injections of toxin into the posterior root lead to a tetanus dolorosus, which is characterized by strictly localized sensitiveness to pain.

Ascending centripetally along the motor paths, the poison reaches the motor spinal ganglia on the side of inoculation; then it affects the ganglia of the opposite side, making them hypersensitive. The visible result of this is the highly increased muscle tonus—*i. e.*, rigidity. If the supply continues, the toxin next affects the nearest sensory apparatus; there is an increase in the reflexes, but only when the affected portion is irritated. In the further course of the poisoning the toxin as it ascends continues to affect more and more motor centers, and also the neighboring sensory apparatus. This leads to spasm of all the striated muscles and general reflex tetanus. Zupink showed that local tetanus did not develop when toxin was injected where it did not come in contact with muscle. He believed, therefore, that the muscle spasm was due to direct action of the toxin on the muscle fibers.

Field in our laboratory has shown that not only tetanus toxin, but diphtheria toxin and inert colloids can be demonstrated in the sciatic nerves after they have been injected subcutaneously or intramuscularly, and after varying periods may be found in the spinal cord. He believes that the toxins are absorbed by way of the lymphatics of the nerves and not by way of the axis-cylinder.

A later experiment of Cernovodeanu and Henni tends to confirm this contention. They ligated all the muscles and bloodvessels in a guinea-pig's leg, leaving intact only the sciatic nerve, skin and bone, and then injected a large amount of tetanus toxin below the point of ligation. The animals in which this was done never developed tetanus. In this case there was only a very slight flow of lymph into the ligated area, and so there could be only a slight flow up the nerve.

If the toxin gets into the blood the only path of absorption to the central nervous system is still by way of the motor-nerve tracts. There seems to be no other direct path, as, for example, by means of the bloodvessels supplying the central nervous system. Even after introducing the poison into the subarachnoid space, owing to the passage of the poison into the blood, there is a general poisoning and not a cerebral tetanus. This at least is the case if care has been taken during the operation to avoid injuring the brain mechanically. A very much smaller amount of toxin is required to produce fatal tetanus if it is injected into a nerve than if it is injected into the blood.

**The Union of Toxin with the Gray Matter of the Brain and Spinal Cord.**—This union is a loose one and the toxin can be partially freed from its union by action of proteolytic ferments. A number of different elements of the cell substances seem to have this power of binding the toxins. Heating to 65° C. for ten minutes destroys the capacity to fix toxin. These brain substances which unite with toxin are certainly not of the nature of antitoxin, and the brain cells if they produce antitoxin at all certainly share the power with other cells that have no power to bind toxin. Marie, in a recent communication, notes that adrenalin neutralizes tetanus toxin and that lecithin compounds are undoubtedly concerned in the mechanism of tetanus toxin in nerve cells.

**Rapidity of Absorption and Loss of Tetanus Antitoxin from Tissues.**—The absorption of antitoxin administered subcutaneously takes place rather slowly. In his animal experiments Knorr found the maximum quantity in the blood only after twenty-four to forty-eight hours. From that time on the amount again steadily decreased, so that by the sixth day only one-third the optimum quantity was present. By the twelfth day only one-fiftieth and at the end of three weeks no antitoxin whatever could be demonstrated. We injected one of our laboratory assistants with 10,000 units of tetanus antitoxin subcutaneously. The blood antitoxic strength was found to be as follows: 18 hours, 0.6 unit; 24 hours, 0.8; 48 hours, 1; 72 hours, 1; 148 hours, 0.7. The two important facts to be noted are the slow absorption of antitoxin from the subcutaneous tissues and its long retention in the blood. The two charts showing the absorption and disappearance of diphtheria antitoxin apply equally to tetanus antitoxin (see Figs. 209 and 210, Part III).

When injected intravenously the antitoxin very quickly passes into the lymph. Ransom, in 1901, was able to demonstrate it in the thoracic duct of a dog fifteen minutes after intravenous injection. Only after very massive intravenous doses and a considerable interval of time are small traces found in the cerebrospinal fluid. This is the reason that passively and actively immunized animals become tetanic if the poison is injected directly into the central nervous system or into a peripheral nerve. Antitoxin injected subdurally passes almost entirely over into the blood within twenty-four hours.

So long as the toxin circulates in the blood it is neutralized by antitoxin in about the same proportion as in test-tube experiments. By means of intravenous injections of antitoxin Ransom was able to render the lymph free from toxin in a very few minutes. According to Marie and Morax, toxin injected into the muscles is already demonstrable in the nerve tissue at the end of one and a half hours—*i. e.*, it has already entered the channel, where it is reached with difficulty by the antitoxin. Donitz injected various rabbits intravenously, each with 1 c.c. of a toxin solution containing twelve fatal doses. Thereupon he determined the dose of antitoxin which, when intravenously given, would neutralize this poison after various intervals of time The antitoxin was of such a strength that in test-tube experiment 1 c.c. of a 1 to 2000 solution just neutralized the amount of toxin employed. He found that at the end of two minutes double the dose required *in vitro* would still neutralize the poison; at the end of four minutes about four times the dose was required, and at the end of eight minutes ten times. When one hour had been allowed to elapse forty times the original dose just sufficed to protect the animal from death, but not from sickness.

**Differential Diagnosis between Tetanus and Tetanus-like Bacilli.**—The differential diagnosis of the bacillus of tetanus is, generally speaking, not difficult, inasmuch as animal inoculation affords a sure test of the specific organism. No other microörganism known produces effects similar to the tetanus bacillus, nor is any other neutralized by tetanus antitoxin. The other characteristics also of this bacillus are usually distinctive, though microscopic examination alone cannot be depended on to make a differential diagnosis. Difficulty arises when other anaërobic, or aërobic bacilli, almost morphologically identical with the tetanus bacillus, are encountered which are non-pathogenic, such as the Bacillus pseudotetanicus (anaërobius), and the Bacillus pseudotetanicus (aërobius). It is possible, however, that both these bacilli, when characteristic in cultures, are only varieties of the tetanus bacillus, which, under unfavorable conditions of growth, have lost their virulence. These non-virulent types do not, as a rule, have spores absolutely at their ends, and the spores themselves are usually more ovoid than those in the true tetanus bacilli.

**Methods of Examination in a Case of Tetanus.**—(*a*) *Microscopic.*—From every wound or point of suppuration film preparations should be made and stained with the usual dyes. The typical spore-bearing forms are looked for, but are usually not found. At the same time other bacteria are noted if present.

(*b*) *Cultures.*—Bits of tissue, pus, cartridge wads, etc., are collected and dropped into glucose bouillon preferably in fermentation tube with a piece of tissue, etc. (see Isolation).

(*c*) *Inoculation.*—A salt solution emulsion of material from the wound is inoculated into mice or guinea-pigs subcutaneously. Successful results are usually obtained, if the bacilli or their spores are present.

## BACILLUS (ANTHRACIS) SYMPTOMATICI (BACILLUS OF SYMPTOMATIC ANTHRAX).

The bacillus of symptomatic anthrax is the cause of the disease in animals—principally cattle and sheep—known as "black leg," "quarter evil," or symptomatic anthrax (rauschbrand, German; charbon symptomatique, French), a disease which is characterized by a peculiar emphysematous swelling of the subcutaneous tissues and muscles, especially over the quarters. Clinically it is sometimes confused with anthrax. Like B. anthrax, it is an inhabitant of the soil and is a large spore-bearing organism, but it grows only under anaërobic conditions and is otherwise distinctly different from B. anthracis.

**Morphology.**—Bacilli having rounded ends, from 0.5$\mu$ to 0.6$\mu$ broad and from 3$\mu$ to 5$\mu$ long, mostly isolated, also occurring in pairs, joined end-to-end, but never growing out into long filaments, as the anthrax bacilli in culture and the bacilli of malignant edema in the bodies of animals are frequently seen to do. The bacilli are actively motile. The spores are elliptic in shape, usually thicker than the bacilli, lying near the middle of the rods. This gives to the bacilli-containing spores a somewhat spindle shape. It stains readily with the ordinary dyes and is Gram-amphophile.

**Biology.**—It is a strict anaërobe, developing at room temperature better at 37° C. It grows best in albuminous or tissue media and when glucose is present. It ferments this sugar with the production of gas. Gelatin is liquefied.

**Growth on Agar.**—The colonies on agar are somewhat more compact than those of malignant edema, but also send out filamentous projections.

**Pathogenicity.**—The bacillus of symptomatic anthrax is pathogenic for cattle, sheep, goats, guinea-pigs, and mice. The guinea-pig is the most susceptible of test animals, and cultures from the heart blood of inoculated guinea-pigs offer the best method of obtaining pure cultures. When susceptible animals are inoculated subcutaneously with pure cultures of this organism, or with spores attached to a silk thread, or with bits of tissue from the affected parts of another animal dead of the disease, death ensues in from twenty-four to thirty-six hours. At the autopsy a bloody serum is found in the subcutaneous tissues, extending from the point of inoculation over the entire surface of the abdomen, and the muscles present a dark red or black appearance, even more intense in color than in malignant edema, and there is a considerable development of gas. The lymphatic glands are markedly hyperemic.

The disease occurs chiefly in cattle, more rarely in sheep and goats; horses are not attacked spontaneously—*i.e.*, by accidental infection. In man infection has never occurred. The usual mode of natural infection by symptomatic anthrax is through wounds which penetrate not only the skin, but the deep, intercellular tissues; some cases of infection by ingestion have been observed. The pathological findings present the conditions above described as occurring in the experimental animals.

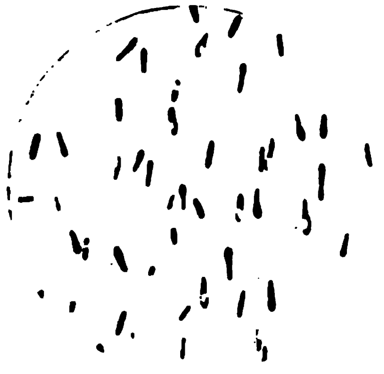

FIG. 161.—Bacilli of symptomatic anthrax showing spores. (After Zettnow.)

**Distribution Outside of the Body.**—Symptomatic anthrax, like anthrax and malignant edema, is a disease due to soil infection, being confined especially to places over which infected herds of cattle have been pastured. By contamination of deep wounds acquired by animals in infected pastures, the disease is spread. The spores are extremely resistant.

**Toxins.**—Under favorable conditions extracellular toxins are formed. Injections of the toxin into animals excite the production of antitoxins.

**Preventive Inoculations.**—Recovery from one attack of symptomatic anthrax protects an animal against a second infection. Active immunity can be produced by vaccines of attentuated organisms. A dried powder of the muscles of animals which have succumbed to the disease is used as a vaccine and subjected to a suitable temperature to insure attenuation of the virulence of the spores contained therein. Two vaccines are prepared—a stronger vaccine by exposing a portion of the powder to a temperature of 85° to 90° C. for six hours, and a weaker vaccine by exposing it for the same time to a temperature of 100° to 104° C. Inoculations are made with this attenuated virus into the end of the tail —first the weaker and later the stronger. The results obtained from this method of preventive inoculation seem to have been very satisfactory.

## THE MALIGNANT EDEMA BACILLUS OR VIBRION SEPTIQUE.

Bacilli of this group are widely distributed, being found in the superficial layers of the soil, in putrefying substances, in foul water. This bacillus was discovered (1876) by Pasteur in animals after infection with putrid flesh, and named by him "vibrion septique." He did not obtain it in pure culture. Koch and Gaffky (1881) carefully studied this microörganism, described it in detail, and gave it the name "Bacillus edematis maligni" (Fig. 162).

**Morphology.**—The edema bacillus is a rod of from $0.8\mu$ to $1\mu$ in width, and of very varying length, from $2\mu$ to $10\mu$ or more, according to the conditions of its cultivation and growth. It is usually found in pairs, joined end to end, but may occur in chains or long filaments. It is motile, and does not produce a capsule. It forms spores which are situated in or near the middle of the body of the rods, exceptionally near the ends. The spores vary in length and are oval in form, being often of greater diameter than the bacilli, to which they give a more or less oval shape.

The bacilli *stain* readily by the usual anilin colors employed and are Gram-amphophile.

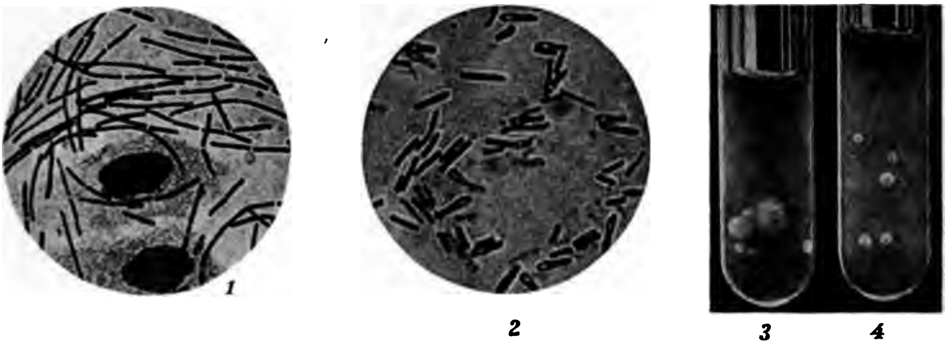

FIG. 162.—Bacilli of malignant edema. 1, bacilli; 2, with spores; 3, and 4 deep colonies in dextrose nutrient agar. (Kolle and Wassermann.)

**Biology.**—An obligate anaërobe growing best on or in albuminous media but also growing well on ordinary media especially if available carbohydrates are present. Growth occurs at 20° C., but is more rapid and abundant at 37° C.

**Growth on Agar.**—On dextrose agar plates the colonies appear as dull, whitish points, irregular in outline, and when examined under a low-power lens are seen to be composed of a dense network of interlacing threads, radiating irregularly from the center toward the periphery.

**Growth in Gelatin.**—The colonies are similar to those on agar, a liquefied zone developing after several days.

**Resistance.**—The spores are very resistant and because of this the soil remains infected.

**Other Media.**—Milk is coagulated and then digested. Blood serum is liquefied. An offensive odor develops due to the proteolysis: Glucose is fermented with the production of acid and gas.

**Pathogenicity.**—Malignant edema is ordinarily confined to domestic animals, horse, sheep, cattle and swine. It follows the contamination of wounds with infected soil or other infectious material; and also occurs as a complication of surgical operations. It is therefore a frequent contaminant after war wounds. The depth of the wound as well as the introduction of foreign particles and other bacteria are factors in infection, as the inoculation of washed spores frequently fails to produce infection. An extensive hemorrhagic edema of the subcutaneous tissues develops from the site of the wound. The serous effusion is frothy from gas production and has a foul odor. As a rule in the larger animals the bacilli do not invade the blood until after death. The bacillus is pathogenic for the smaller laboratory animals, rabbit, guinea-pig, mouse, a septicemia developing as well as the local edema.

**Immunity.**—Recovery from infection is followed by immunity Weinberg and Seguin[1] have demonstrated a toxin and have produced a serum claimed to be effective in helping to prevent gaseous gangrene (See Part III).

**Bacillus Tumefaciens.**—Wilson[2] has described under this name an organism which he says is a new species found in a case of gas gangrene following a war wound. It resembles Vibrion septique, but it is much more active in all of its reactions.

## BACILLUS EDEMACIENS.

This organism was isolated by Weinberg and Seguin as a new species. They describe it as follows: It is rather a stout bacillus, occurring singly or in long chains. It is Gram-positive and practically non-motile in ordinary media. It possesses very many flagella. It sporulates in all media, even when glucose is added. The spores are usually subterminal. It is a very strict anaërobe, growing with difficulty. This is the reason why so many workers have missed it.

The colonies in deep, nitrated agar are at first opaque in the center and surrounded by unequal filaments. Later, they become clearer, the center cloudy, with a periphery of hairy filaments (very much like tetanus or Vibrion septique colonies).

In Martin's broth the growth is first slightly cloudy, then the broth becomes perfectly clear, a precipitate settling at the bottom. This bacillus ferments glucose, maltose, lactose, saccharose, glycerin and starch. Acid production is not great, though there is considerable gas production. It does not liquefy coagulated horse serum. It secretes a very active soluble toxin which stimulates the production of antiserum. Antitoxic titer 4000 units, that is, $\frac{1}{4000}$ c.c. neutralizes 100 fatal doses of the toxin.

Dr. Weinberg found other strains similar to their own culturally and morphologically, but not at all pathogenic. Their strains are very virulent for the laboratory animals and produce a clear, gelatinous edema.

**Bacillus Bellonensis.**—A form described as similar to B. edemaciens and possibly only a variant.

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[1] La gangrène gazeuse. Masson et Cie., Paris, 1918.

[2] The Lancet, April 19, 1919, p. 657.

## BACILLUS WELCHII GROUP (BACILLUS (AËROGENES) CAPSULATUS, BACILLUS PERFRINGENS).

The first bacillus of this group to be described minutely was found by Welch in the bloodvessels of a patient suffering with aortic aneurysm; at autopsy, made in cool weather, eight hours after death, the vessels were observed to be full of gas bubbles. Since then it has been found in many cases in which gas has developed from within sixty hours of death until some hours after death. It occurs most frequently after external cutting operations and wounds. These cases are, as a rule, marked by delirium, rapid pulse, high temperature, and the development of emphysema and discoloration of the diseased area or of marked abdominal distention when the peritoneal cavity is involved. Members of this group are present, as a rule, in the intestinal canal of man and animals and are apt to be found in the dust of hospitals and elsewhere. Herter[1] has shown that they are present in excessive numbers in certain diseases of the digestive tract. These cases are apt to develop anemia.

Different strains of bacilli belonging to this group have appeared under different names and their exact relationship is still uncertain. Thus B. phlegmonis emphysematosæ of Fränkel is probably the same as B. welchii. B. perfringens (Veillon and Zuber, 1898) and B. enteritidis sporogenes (Klein, 1895) are closely related if not the same organism.[2]

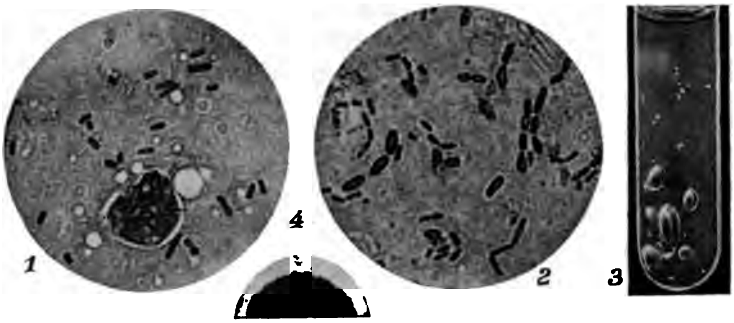

FIG. 163.—Bacillus aërogenes capsulatus. 1, bacilli, 2, spores; 3, culture in dextrose-nutrient agar.

**Morphology.**—Straight or slightly curved rods, with rounded or sometimes square-cut ends; somewhat thicker than the anthrax bacilli and varying in length; occasionally long threads and chains are seen. The bacilli in the animal body, and sometimes in cultures, are enclosed in a transparent capsule. Spores are usually absent in the tissues and often in cultures. Dunham showed that the culture isolated by Welch formed spores when grown on blood serum. Some strains since isolated make spores readily.

**Biology.**—Anaërobic, non-motile, non-liquefying bacilli. They are positive to Gram, but are more easily decolorized than many bacteria.

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[1] Jour. Biol. Chem., 1906, ii, 1.

[2] Simonds: Jour. Inf. Dis., 1915, i, 31.

Growth is rapid at 37° C., in the usual culture media containing certain sugars, and in the absence of oxygen, and is accompanied by the production of gas. *Nutrient gelatin* is not liquefied, but it is gradually peptonized. On *agar* colonies are developed, which are from 1 to 2 mm. or more in diameter, grayish-white in color, and in the form of flattened spheres, ovals, or irregular masses, covered with hair-like projections. *Bouillon* is diffusely clouded, and a white sediment is formed. *Milk* becomes acidified and coagulated, then partially digested, giving a worm-eaten appearance to the clot. A large amount of butyric acid is produced.

**Isolation.**—When quantities up to 2.5 c.c. of fresh bouillon cultures are injected into the circulation of rabbits and the animals killed shortly after the injection, the bacilli develop rapidly, with an abundant formation of gas in the bloodvessels and organs, especially the liver. This procedure is one of the best methods of obtaining the bacilli. The material suspected of containing the bacillus alone or associated with other bacteria is injected intravenously into rabbits, which are killed five minutes later and kept at 37° C. for sixteen hours, and cultures made from the liver and heart blood.

**Pathogenicity.**—Its pathogenicity is usually not marked in healthy animals, although Dunham found that the bacillus taken freshly from human infection is sometimes very virulent.

As we have said, bacilli of this type are one of the frequent infections after irregular unclean wounds such as those received in war. In these infections there is marked destruction of tissue, especially in the muscles. This has been supposed to be due to one or more of five conditions: (1) to the large quantity of butyric acid produced from the glycogen of the muscles; (2) to pressure from the amount of gas produced; (3) to presence of foreign protein; (4) to specific toxin; (5) to the presence of a soluble, ionizable *calcium* salt which, according to Bullock and Cramer,[1] produces a local breakdown of the normal defensive mechanism against spore-bearing anaërobes. Many attempts have been made to demonstrate a specific toxin production by these bacilli (Harde,[2] Weinberg,[3] Bull and Pritchett[4]), and the last three have demonstrated that an exotoxin is produced which is controlled by an antitoxin. Robertson[5] has made a study of the isolation and types of anaërobes found in wounds.

It is suggested by Welch that in some of the cases in which death has been attributed to the entrance of air into the veins the gas found at the autopsy may not have been atmospheric air, but may have been produced by this or some similar microörganism entering the circulation and developing shortly before and after death. The same may be true for gas in the uterine cavity.

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[1] Proc. Roy. Soc., London, 1919, xci, 513.

[2] Comp. rend. Soc. de Biol., 1915, lxxviii, 134.

[3] Proceed, Roy. Soc. Med., 1916, ix, 119.

[4] Jour. Exp. Med., 1917, cvi, 825.

[5] Jour. Path. and Bact., 1916, xx, 327.

## WAR WOUNDS.

During the recent great war many studies have been made on the bacteriology of war wounds and the part the laboratory plays in an early diagnosis and in aids for treatment. These studies may be summarized as follows:

The number and kinds of bacteria found in war wounds depend chiefly upon the time elapsing between the hour when the patient is wounded and the hour when the examination of the wound is made. Practically no organisms can be demonstrated in cultures until at least four hours or in smears until at least six hours after the wound is made. After that, several varieties of both anaërobic and aërobic bacteria develop quickly in untreated infected wounds.

The varieties most to be feared among the anaërobes, according to the latest opinion, are Vibrion septique, B. welchii and B. edemaciens. A number of other varieties (*e. g.*, B. hemolyticus, B. bellonensis, B. fallax, "Reading bacillus," B. egens) have been described as helping in the production of "gas gangrene," but they have been less frequently met, and their role may be only a secondary one. All of these anaërobes are most frequently found in varying combinations, almost never alone. In the rare infections in civil practice the B. welchii is sometimes met with in pure culture. A classification of the chief war wound anaërobes is given by McIntosh.[1]

Among the aërobes the most to be feared is St. hemolyticus. Many other aërobes have been found as secondary infections in war wounds; for example, staphylococci, St. viridans, enterococcus, B. pyocyaneus, B. proteus, B. coli, B. diphtheriæ, diphtheroid bacteria. These probably aid the development of the anaërobes.

There are two practical reasons for making a bacteriological diagnosis in an open wound. The first is to determine the type of anaërobe present in order to give specific serum. Serums which are specific have been produced against Vibrion septique, B. welchii, B. edemaciens respectively (see Part III), and Sacquepée[2] recommends the following procedure for the differentiation.

> In each of four test-tubes is placed 1 c.c. of the macerated gangrenous tissue and to three tubes, respectively, is added 1 c.c. of each of the three antiserums. After half an hour's incubation the contents of each tube is injected respectively into one of four guinea-pigs, according to Sacquepée. The one protected by the serum shows no reaction; the others die. They usually become sick in from six to twelve hours.

The second reason for making a bacteriological diagnosis is in order to inform the surgeon as to the possibility and the time for performing a primary, delayed primary or a secondary closure. The bacteriological examination of recent injuries, to be of most aid to the surgeon (according to Beebe[3]), should be made after the primary operation as follows:

[1] Publication of Medical Research Com., London, 1917, p. 74.

[2] Sacquépée and de la Vergne: de l'Acad. de Méd., 1919, lxxxi, 504.

[3] Wound Bacteriology. War Medicine, January, 1919, p. 1023,

A few strands of silkworm gut or horse-hair should be placed at the most dependent part of the wound. At the first dressing, twenty-four hours later, these should be removed and from the serum that exudes the bacteriologist should make his cultures and smears. The examination of open wounds should be made from material curetted from the deeper pockets.

If the count in the stained spreads shows only one bacterium in two fields, or less, for two successive counts and the cultures show no hemolyzing cocci and very few anaërobic bacilli the wound may be closed. If hemolytic streptococci have been shown to be present the wound should not be closed until several repeated cultures show the absence of hemolytic streptococci. Considerable numbers of staphylococci and of bacilli of the colon group sometimes prevent primary union. The counting of the number of bacteria in the cover-glass is not an accurate procedure, but suffices for guidance when controlled by cultures.

## BACILLUS BOTULINUS.

This bacillus, while not pathogenic for man as an infectious organism, is, as first shown by Van Ermengen, the causative agent in a characteristic type of food poisoning, the symptoms being due to the toxin produced by the growth of this bacillus in foods. The clinical symptoms of this type of food poisoning are neuroparalytic in character. There are secretory disturbances, as increase or suppression of the salivary secretions or a thick secretion of mucus in the mouth and pharynx. Disturbances of accommodation, ptosis, double vision, and dysphagia are the common motor symptoms. Obstinate constipation and retention of urine as well as disturbances in heart action and respiration occur. Fever is absent. Death is not uncommon and is due to respiratory paralysis. Graham and Brueckner[1] have recently reported results indicating that the toxin of this bacillus or similar bacilli is the causative agent in some outbreaks of forage poisoning or so-called cerebrospinal meningitis in horses.

**Morphology.**—The bacilli are large, 4 to $6\mu$ long and 0.9 to $1.2\mu$ wide, with somewhat rounded ends. They are slightly motile, Gram-positive, and have oval spores, usually terminal. Short chains may be produced.

**Biology.**—It is anaërobic, growing best at 22° to 25° C. Gelatin is liquefied and glucose fermented with the production of acid and gas. A butyric acid odor develops in cultures. The colonies on gelatin are yellowish, translucent, coarsely granular, the granules moving slowly when liquefaction begins. The older colonies are brownish, more opaque and show fine thorn-like extensions.

**Strains.**—There are at least two distinct strains.

**Resistance.**—The spores are moderately resistant to heat, being killed in fifteen minutes at 90° C. or in one-half hour at 85° C.

**Toxin Production.**—A thermolabile extracellular toxin is produced in glucose-broth cultures. Guinea-pigs, rabbits, mice, cats and monkeys are susceptible to the toxin and succumb not only to injection but also when the toxin is given by mouth. As little as 0.0001 c.c. may produce symptoms. After an incubation period, dyspnea, convulsions and paralysis occur and death is due to respiratory paralysis. The paralyses have been attributed to degeneration of the ganglion cells of the anterior horn and of the bulbar centers. According to Dickson[1] this is secondary to disturbances in blood supply due to thrombosis associated with meningeal hemorrhage. He suggests the possibility that the toxin acts on the terminal end-plates. An antitoxin has been produced.

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[1] Jour. Bact., 1919, iv, 1.

**Foods Involved.**—Meat and meat preparations, such as canned or pickled meats and sausages are the usual offending foods. Until recently it was thought that preserved or canned fruits or vegetable products were not factors. Cases due to such foods have been reported and Dickson has shown that the toxin develops in a medium of peas or beans. All these foods have one thing in common, they are prepared for weeks or months before they are consumed. The source of contamination is not definitely known, though the organism has been isolated from pigs' feces.

**Diagnosis of Botulism.**—The diagnosis usually rests on the clinical manifestations. Verification may be attempted by examination of the suspected food. A thick emulsion of the food is made in saline and from it glucose gelatin plates and fermentation tubes (with tissue) are inoculated. A portion of the suspension can be heated (60° C., one-half hour) and similarly inoculated. From the growth in the fermentation tube, heated and unheated anaërobic plates are made. The pure cultures obtained are then tested for toxin production by animal inoculation. Saline extracts of the suspected food, if they produce characteristic symptoms in experimental animals, confirm the clinical diagnosis in man. Intoxication is more common from foods prepared in the west than in the east.

**Prophylaxis.**—Although a rancid butter odor may be present in contaminated foods, it may be slight and not recognized. Cooking will destroy the toxin. All canned material should be heated to boiling before use. Cleanliness in preparation is an aid in preventing contamination and also in limiting the numbers of associated bacteria which, by their growth, would aid in establishing anaërobic conditions. Brine for pickling should contain at least 10 per cent. of salt as this concentration prevents the growth of the bacilli. Incomplete sterilization is the source of danger with canned goods.

## BACILLUS FUSIFORMIS (BACILLUS OF VINCENT'S ANGINA).

This organism together with spirochetes was found by Plaut and Vincent in pseudomembranous inflammation of the throat (Vincent's Angina). The constancy of its presence and the preponderance of their numbers in smears from this condition suggest strongly their etiological connection with the disease. Fusiform bacilli and spirochetes are also encountered in gangrene, noma, ulcerative stomatitis, gingivitis, dental caries and even around the gum margins of dirty teeth, especially if there are deposits of tartar. Although B. fusiformis is present in many conditions, either because of the greater virulence of certain strains or because of reduced resistance of the tissues they, together with the spirochetes are probably the essential agent in the production of Vincent's angina and noma and act as a contributing factor in other lesions.

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[1] See Botulism—A Clinical and Experimental Study, Monograph VIII, Roch. Inst. Med. Res., 1918.

The fusiform bacilli are anaërobic and have been cultivated. The most successful methods of isolation and cultivation are those of Krumwiede and Pratt,[1] who studied fifteen strains from various conditions. The typical bacillus is double-pointed, containing one or more granules, which are well demonstrated by Giemsa's stain. In culture the morphology is variable. In fluid media there is a tendency to produce filamentous types, which may form tangled, thread-like masses. The colonies are characterized by thread-like outgrowths. The fifteen cultures studied fell into saccharose fermenting and saccharose non-fermenting groups, but this difference had no correlation to the source of the culture. The fusiform bacillus and the spirochetes accompanying it in the lesion were never encountered together in cultures made from single colonies although Tunnicliff claimed that the fusiform bacillus and the spirochete were only different forms of the same organism.

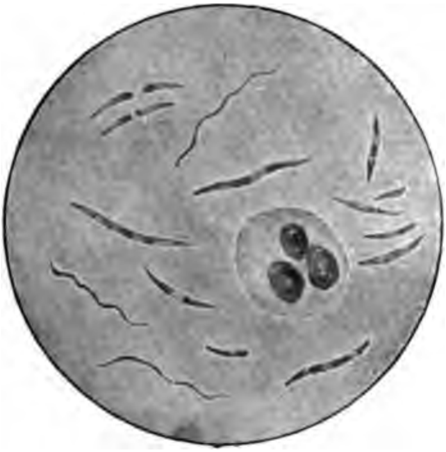

FIG. 164.—Vincent's bacillus with accompanying spirochetes.

The microscopic diagnosis of B. fusiformis may be made in direct smears from the lesion. The differential diagnosis between the short, more deeply staining club-like forms and B. diphtheriæ, as it often appears in direct smears stained by alkaline methylene blue solution, may be made by staining by Gram's stain, which shows B. fusiformis, as mostly negative to Gram, with faintly staining granules.

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[1] Jour. Inf. Dis., 1913, xii, 199, and xiii, 438.

## "BACILLUS TYPHI-EXANTHEMATICI."

**Historical.**—The "Bacillus typhi-exanthematici" was first isolated and named by Plotz,[1] in 1914, from the blood of individuals suffering from typhus fever. A year later, Plotz, Olitsky and Baehr[2] published the report of an extensive study of bacteriological, serological and animal investigations which they claimed contained evidence that this organism is the causative agent in typhus fever. Investigations by others have made the evidence weaker (see under Filtrable Viruses). The following description of the bacillus is from the reports of Plotz and his co-workers.

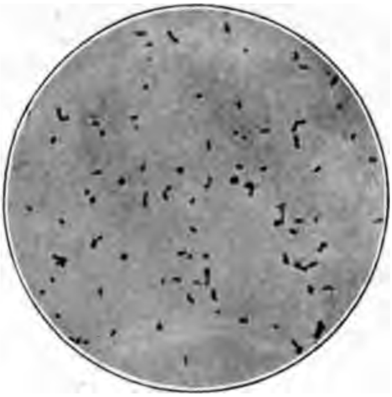

FIG. 165.—Typhus bacillus. (Plotz.)

**Morphology.**—The organism is a small, slender bacillus, the average length being about one micron. In young cultures the organisms are small and uniform in size; in very old subcultures there is often an admixture of various sized bacilli from coccoid forms up to some measuring two microns in length. The bacilli are usually straight, though slightly curved forms occur occasionally. The ends are slightly pointed, less often rounded. (Fig. 165.) With special stains an occasional organism will show a fine polar body at one end, more rarely at both.

**Reaction to the Gram Stain.**—The organism in subcultures is Gram-positive. According to Olitsky, Denzer and Husk[3] and Baehr and Plotz,[4] the colonies in occasional blood cultures may consist only of exceedingly minute bacilli which are completely decolorized by Gram's method. In subsequent subcultures these bacilli always become Gram-positive. A similar experience was the rule in organisms isolated from typhus infected lice.

**Cultural Characteristics.**—The bacillus typhi-exanthematici when first isolated is an obligate anaërobe. After artificial cultivation for many months, it has been possible to obtain slight aërobic growth with occasional strains.

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[1] Jour. Am. Med. Assn., 1914, lxii, 1556; La Presse Med., 1914, xliii, 411.

[2] Jour. Inf. Dis., 1915, xvii, 1.

[3] Jour. Am. Med. Assn., 1916, lxvi, 1692; Jour. Inf. Dis., 1916; 1917, xv, 99 and 349.

[4] Jour. Inf. Dis., 1917, xx, 201.

The organism, as far as tested, grows only upon a medium containing ascitic or hydrocele fluid and glucose. Although growth occurs in fluid media containing these ingredients, the optimum medium contains 1 part of ascitic fluid and 2 parts of 2 per cent. glucose agar. Even on slants of the medium a large amount of culture material must be subinoculated in order to obtain growth and it is advisable to smear it in thick streaks upon the surface. The subcultures upon such slants incubated in Buchner tubes containing equal parts of pyrogallic acid and 30 per cent. sodium hydrate solution usually show evidences of growth after about three days. The growth is profuse by the sixth or seventh day and at that time is soft and creamy in character, raised above the surface and of a glistening white color. In the medium itself a diffuse clouding gradually develops as the growth increases (the precipitation phenomenon of Libman).

Strains of the Bacillus typhi-exanthematici isolated in the United States, Mexico, Serbia and Russia are identical in their action upon sugars. They invariably ferment glucose, maltose, galactose and inulin with marked acid production and precipitation of protein, but have no action upon saccharose, lactose, raffinose, arabinose, dextrin and mannit.

**Method of Isolating the Organism from the Blood.**—The method used in making the anaërobic blood culture is that of Liborius-Veillon, using the ascitic fluid glucose agar recommended by Libman as the optimum medium for bacterial cultivation.

The essential ingredient of the medium is ascitic or hydrocele fluid. It must be clear, free of bile or blood pigment and should possess a specific gravity of more than 1015. Filtered ascitic fluid or one which contains a preservative or has been sterilized by heating should not be used. Pleural fluids are probably useless.

The technic of the culture consists in withdrawing with a syringe and needle 15 c.c. of blood from a vein and dividing this among 8 large test-tubes (20 x 2 cm.) half-filled with melted 2 per cent. glucose agar. To each tube in turn, ascitic or hydrocele fluid equivalent to at least one-third of the volume of the agar (*i. e.*, 6 to 10 c.c.) is added. The contents are then poured once or twice into another sterile test-tube in order to secure thorough mixing. After thorough solidification of the mixture, each tube is covered with a layer of plain agar, 2 or 3 cm. deep, and then the cotton stopper paraffined. The tubes are then incubated at 37° C.

**Appearance of Colonies.**—Colonies usually appear in the culture tubes in about nine or ten days. Occasionally they may occur as early as the fifth or as late as the nineteenth day. They first appear as small opaque spots which, by direct light, are white. During the subsequent two or three days they grow rapidly larger, usually assuming a "Y" shape, and develop a brownish zone of precipitation in the medium about them. In consistency the growth is always exceedingly soft, and the color, especially of the older colonies, is pale brown.

The organism, according to the original authors, may be isolated from the blood during the entire febrile course of the disease, from the first to the last day. Others have not been able to corroborate this. In two cases Plotz was still able to grow the bacillus from the blood twelve and thirty-six hours respectively after the crisis. In the mild cases only one to three colonies may develop in the eight tubes; in severe cases as many as ten to twenty may be present. During chills which occur frequently at the onset of the illness and occasionally during its first week numerous colonies develop from the blood indicating that enormous numbers of the bacteria may be present.

The percentage of positive blood cultures also varies with the severity of the disease, as can be seen from the following table taken from an article by Baehr and Plotz:

| Type of disease. | Mortality per cent. | Number of cases studied. | Percentage with positive blood cultures per cent. |
|---|---|---|---|
| New York typhus (Brill's disease) | 0.2 | 34 | 5 |
| Russian typhus . . . . . | 5.5 | 24 | 79 |
| Mexican typhus . . . . . | 20.0 | 8 | 100 |
| Balkan typhus, 1914 . . . | 18 to 60 | 7 | 100 |

The organisms, the original authors say, can also be pretty regularly isolated from monkeys and guinea-pigs in which the disease has been produced by the inoculation of typhus blood.

**Pathogenicity.**—On artificial media the organism loses its pathogenicity very rapidly. Organisms which have been grown on artificial media for less than a week are sometimes still feebly pathogenic for guinea-pigs but those cultivated for longer periods are always nonpathogenic. After an incubation period more irregular than of typhus fever, the animals may develop a febrile illness during which in several cases the bacillus has been recovered from the blood.

**Development of Antibodies after Typhus Fever.**—Specific agglutinins, precipitins, opsonins and complement-fixing antibodies for the bacillus are regularly present in the blood of typhus convalescents. They usually appear during the second week of the disease, increase as the crisis is approached, reach their maximal titer during the first or second week of convalescence and usually persist in the blood for months, in one case for two and one-half years. The curve of the course of development of the antibodies in this disease is typically an immunity curve. This evidence has lost much of its significance since the finding of abundant agglutinins against one of the proteus group of organisms (see Filtrable Viruses), and the finding of antibodies against the Plotz bacillus in cases other than typhus.

**Prophylactic Immunization.**—The repeated subcutaneous inoculation in human beings of vaccine made from these organisms is followed by the development in their blood of specific antibodies. In guinea-pigs, however, although they develop immunity after an infection due to the blood, such an antibody production does not occur and thus far it has also been found impossible to confer immunity upon these animals by vaccination. Plotz and Baehr carried out investigations in the Balkans and in Volhynia,[1] Russia, where typhus fever was epidemic, upon the value of prophylactic immunization in human beings, and reported that vaccination with Bacillus typhi-exanthematici markedly reduces the incidence of the disease. Others have not been able to corroborate this and the inoculations were not carried out extensively during the final years of the war.

**Transmission by the Body Louse.**—The stomach of typhus infected lice contains immense numbers of bacilli which are morphologically identical with the Bacillus typhi-exanthematici. Such bacilli have been seen in typhus lice in Mexico by Ricketts and Wilder[1] and Olitsky, Denzer and Husk.[2] In smear preparations made from typhus lice, these bacillary organisms are decolorizable by Gram's method. Olitsky, Denzer and Husk succeeded in cultivating them from the lice by the anaërobic method described above, and found that after several generations on artificial media the bacilli gradually became Gram-positive. The bacilli were then morphologically, culturally and serologically identical with the Bacillus typhi-exanthematici and in the early subcultures were pathogenic for guinea-pigs.

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[1] Jour. Inf. Dis., 1917, xx, 201.

**Relation to Typhus Fever.**—With the exception of those who first discovered the bacillus, the majority of bacteriologists regard it as a secondary invader, and believe the primary virus is still unknown.

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[1] Jour. Am. Med. Assn., 1910, liv, 1373.

[2] Ibid., 1916, lxvi, 1692; Jour. Inf. Dis., 1917, xx, 99 and 349.

# CHAPTER XXXIV.

# THE CHOLERA SPIRILLUM (CHOLERA VIBRIO) AND SIMILAR VARIETIES.

In 1883 Koch separated a characteristically curved organism from the dejecta and intestines of cholera patients—the so-called "*comma bacillus.*" It was absent from the blood and viscera, and was found only in the intestines; and the greater the number, it was said, the more acute the attack. Koch also demonstrated an invasion of the mucosa and its glands. The organisms were found in the stools on staining the mucous flakes or the fluid with methylene blue or fuchsin, and sometimes alone; by means of cultivation on gelatin they were readily separated from the stools. Numerous control observations made upon other diarrheic dejecta and upon normal stools were negative; the comma bacillus was found in choleraic material only, or occasionally in small numbers in the stools of healthy persons who came in contact with cholera. Soon, however, other observers described comma-shaped organisms of non-choleraic origin. Finkler and Prior, for instance, found them in the diarrheal stools of cholera nostras, Deneke in cheese, Lewis and Miller in saliva. All of these organisms, however, differed in some respects from Koch's comma bacillus, *and it has since been proved that none of them is affected by the specific serum of animals immunized to cholera.* After a time, therefore, the exclusive association of Koch's vibrio with cholera or those in contact with it became almost generally acknowledged, until now it is regarded by bacteriologists everywhere to be the specific cause of Asiatic cholera. Certain sporadic cases of cholera-like disease, however, are undoubtedly due to other organisms.

**Morphology.**—Curved rods with rounded ends which do not lie in the same plane, of an average of $1\frac{1}{2}\mu$ in length and about $0.4\mu$ in breadth. The curvature of the rods may be very slight, like that of a comma, or distinctly marked, particularly in fresh unstained preparations where the adhesion of two individuals presents the appearance of a half-circle. By the inverse junction of two vibrios S-shaped forms are produced. Longer forms are rarely seen in the intestinal discharges or from the cultures grown on solid media, but in fluids, especially when grown under unfavorable conditions, long, spiral filaments may develop. The spiral forms are best studied in the hanging drop, for in the dried and stained preparations the spiral character of the long filaments is often obliterated. In film preparations from the intestinal contents in typical cases it will be found that the organisms are present in enormous numbers, and often in almost pure culture. In old cultures irregularly clubbed and thickened involution forms are frequent, and the presence in the organisms of small, rounded, highly refractile bodies is often noted.

**Staining.**—The cholera spirillum *stains* with the anilin colors usually employed, but not as readily as many other bacteria; a diluted aqueous solution of carbol-fuchsin (1 to 10) is the most reliable staining agent. It is decolorized by Gram's method. The organisms exhibit one long, fine flagellum attached to one end (other spirilla often have two or more end flagella).

**Biology.**—The cholera spirillum is aërobic, liquefying, and extremely motile. It grows readily on ordinary culture media, best at 37° C., but also at room temperature, 22° C.

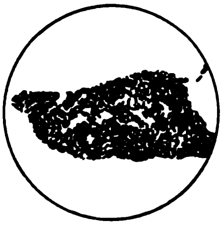


FIG. 166.—Contact smear of colony of cholera spirilla from agar. × 700 diameters. (Dunham.)

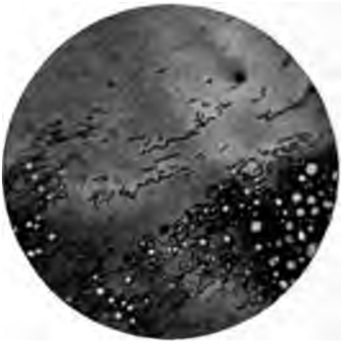


FIG. 167.—Cholera spirilla preparation from gelatin-plate culture of cholera. × 800 diameters.

**Gelatin Plate Cultures.**—In gelatin plate cultures, characteristic colonies are produced. After twenty-four hours' growth, there is a uniform granular appearance of the surface, which because of the high degree of refraction gives the appearance of being powdered with broken glass. Color is practically absent or there may be a slight yellowish tint thus differing from B. coli. If growth is continued, liquefaction appears about the colony and its appearance gradually changes. The characteristic colony in gelatin used to be of the greatest practical importance. With the introduction of special selective media, however, the use of gelatin has been practically discontinued.

**Gelatin Stab Cultures.**—In gelatin stab cultures, a small funnel of liquefaction appears after twenty-four hours. This deepens and broadens, until at the end of a week liquefaction may be complete.

**Agar.**—On agar a moist, shiny, grayish-yellow layer develops. On the surface of alkaline-agar plates the individual colonies are characteristic. They are round, transparent and have a rather distinctive opalescent sheen. This characteristic appearance is made use of for isolation.

On **Dieudonné alkaline-blood agar** the colonies are large and circular. On transmitted light there is a glassy transparency, on reflected light they are grayish. On **alkaline-egg agar** the colonies, when examined by transmitted light, appear to be deep in the agar and have a distinctive hazy appearance due to the development of a halo about the colony. A zone of clearing may develop where growth is continued. As some fecal bacteria other than vibrios may develop on these media, the development of a typical colony is a great aid in isolation.

**Löffler's blood serum** is rapidly liquefied at 37° C. **Milk** is not coagulated. **Glucose** and **saccharose** are fermented with great rapidity, acid only being formed. Growth in fluid media is abundant and usually characteristic, most so in peptone-water. **Peptone-water** is diffusely cloudy to a moderate degree, but at the surface the cloud is much denser. Due to the greater supply of oxygen, the vibrios seek the surface and multiply there more freely.

**Reaction of Media.**—Cholera grows best on media that are strongly alkaline to litmus. They can grow in an excess of alkali sufficient to inhibit the growth of many of the bacteria found in feces. This is most evident where an alkali-albumin mixture is used to increase the alkalinity. (See Special Media.)

**Cholera-red Reaction.**—All cholera strains give this reaction. This is important as many non-cholera spirilla do not give it.

**Changes due to Artificial Cultivation.**—Cholera strains which have been in cultivation for some time show more spiral forms. They grow less typically or not at all on selective media and fluid cultures may develop a wrinkled pellicle. Their digestive powers are also lessened, as gelatin-liquefaction or the liquefaction of Löffler's serum medium. Variations can also be induced by special conditions.

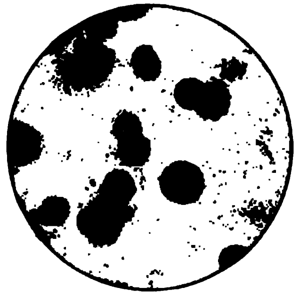


FIG. 168.—Cholera colonies in gelatin; twenty-four to thirty-six hours' growth. × about 20 diameters.

**Hemolysin Production.**—Six strains were isolated by Gottschlich at El Tor from Pilgrims to Mecca, who died with diarrheal symptoms but had shown no clinical evidences of cholera. These strains are called "El Tor" strains. They give the serum reactions of cholera vibrios but produce a strong hemolysin. Kraus and Ruffer believed them not to be true cholera, as hemolysin production was considered by the former as an attribute possessed only by non-cholera types. Further investigations have shown that cholera vibrios may develop, lose, or show wide variations in their ability to produce hemolysins. It is apparently a potential power of all vibrios and of no value in differentiation. For this reason the El Tor strains must be considered as cholera vibrios.

**Resistance and Vitality.—Development Outside of Body.**—If a culture is spread on a cover-glass and exposed to the action of the air at room temperature the spirilla will be dead at the end of two or three hours, unless the layer of culture is very thick, in which case it may take twenty-four hours or more to kill all the spirilla. This indicates that infection is rarely if ever produced by means of dust or other dried objects contaminated with cholera spirilla. The transmission of these organisms through the air, therefore, can only take place for short distances, as by the spray from infectious liquids.

The cholera bacillus is also injuriously affected by the abundant growth of saprophytic bacteria. It is true that when associated with other bacteria, if present in large numbers, and if the conditions for their development are particularly favorable, the comma bacillus may at first gain the upper hand, as in the moist linen of cholera patients, or in soil impregnated with cholera dejecta; but later, after two or three days, even in such cases, the bacilli die off and other bacteria gradually take their place. Thus, Koch found that the fluid contents of privies twenty-four hours after the introduction of comma bacilli no longer contained the living organisms; in impure river water they were not demonstrable for more than six or seven days, as a rule. In the dejecta of cholera patients they were found usually only for a few days (one to three days), though rarely they have been observed for twenty to thirty days, and on one occasion for one hundred and twenty days. In unsterilized water they may also retain their vitality for a relatively long time; thus, in stagnant well water they have been found for eighteen days, and in an aquarium containing plants and fishes, the water of which was inoculated with cholera germs, they were isolated several months later from the mud at the bottom. Koch found them in the foul water of a tank in India, used by the natives for drinking purposes. In running river water, however, they have not been observed for over six to eight days. In milk they are finally destroyed by acidity due to the growth of the milk bacteria, in sterilized milk they may survive eight to ten days. For the cholera organisms the conditions favorable to growth are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material. These conditions are fully met, outside the body, in very few localities.

The comma bacillus has the average resistance of spore-free bacteria, and is killed by exposure to moist heat at 56° C. in half an hour, at 80° in five minutes, at 95° to 100° C. in one minute. The bacilli have been found alive kept after a few days in ice, but ice which has been preserved for several weeks does not contain living bacilli.

Chemical disinfectants readily destroy the vitality of cholera vibrios. For disinfection on a small scale, as for washing the hands when contaminated with cholera infection, a 0.1 per cent. solution of bichloride of mercury, or a 2 to 3 per cent. solution of carbolic acid or, better, lysol may be used. For disinfection on a large scale, as for the disinfection of cholera stools, strongly alkaline milk of lime is an excellent agent. The wash of cholera patients, contaminated furniture, floors, etc., may be disinfected by a solution of 5 per cent. carbolic acid and soap water. For the disinfection of drinking water, chlorinated lime, 1 or 2 parts per million of free chlorine may be used for fairly pure water. Five parts per million would probably be effective even in polluted water.

**Pathogenesis.**—Not one of the lower animals is naturally subject to cholera. Koch succeeded in producing symptoms and intestinal lesions in guinea-pigs similar to those in man by introducing cultures by catheter after neutralizing the contents of the stomach with a solution of carbonate of soda and inhibiting peristalsis by the use of opium. Thomas injected a dilute suspension of cholera spirilla into the ear vein of young rabbits, and thus caused symptoms and lesions of the intestinal mucous membrane resembling those of cholera in man. The spirillum was recovered from the feces. Metchnikoff was successful with suckling rabbits by rubbing a small amount of a culture on the teats of a mother rabbit. Intraperitoneal injections with cholera spirilla kill guinea-pigs acutely, but intestinal lesions are rarely found.

**Accidental Human Infection.**—There are several cases on record which furnish the most satisfactory evidence that the cholera spirillum is able to produce the disease in man. In 1884 a student in Koch's laboratory in Berlin, who was taking a course on cholera, became ill with a severe attack of cholera. At that time there was no cholera in Germany, and the infection could not have been produced in any other way than through the cholera cultures which were being used for the instruction of students. In 1892 Pettenkofer and Emmerich experimented on themselves by swallowing small quantities of fresh cholera cultures obtained from Hamburg. Pettenkofer was affected with a mild attack of cholerine or severe diarrhea, from which he recovered in a few days without any serious effects, but Emmerich became very ill. On the night following the infection he was attacked by frequent evacuations of the characteristic rice-water type, cramps, tympanites, and great prostration. His voice became hoarse, and the secretion of urine was somewhat diminished; this condition lasting for several days. In both cases the cholera spirillum was obtained in pure culture from the dejecta. Finally, there is the case of Dr. Oergel, of Hamburg, who accidentally, while experimenting on a guinea-pig, allowed some of the infected peritoneal fluid to squirt into his mouth. He was taken ill and died a few days afterward of typical cholera, though at the time of his death there was no cholera in the city.

**Lesions in Man.**—Cholera in man is an infective process of the epithelium of the intestine, in which the spirilla clinging to and between the epithelial cells produce a partial or entire necrosis and final destruction of the epithelial covering, which thus renders possible the absorption of the cholera toxin formed by the growth of the spirilla. The larger the surface of the mucous membrane infected and the more luxuriant the development of bacilli and the production of toxin, the more pronounced will be the poisoning, ending fatally in a toxic paralysis of the circulatory and thermic centers. On the other hand, however, there may be cases where, in spite of the large number of cholera bacilli present in the dejecta, severe symptoms of intoxication may be absent. In such cases the destruction of epithelium is not produced or is so slight that the toxic substance absorbed is not in sufficient concentration to give rise to the algid stage of the disease, or for some reason the spirilla do not produce toxin to any extent.

**Distribution in the Body.**—The cholera spirilla are found only in the intestines and are believed never to be present in the blood or internal organs. The lower half of the small intestine is most affected, a large part of its surface epithelium becoming shed. The flakes floating in the rice-water discharges consist mostly of masses of epithelial cells and mucus, among which are numerous spirilla. The spirilla also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial lining, which become loosened by their action. They are rarely found in the connective tissue beneath, and never penetrate deeply. In more chronic cases other microörganisms play a greater part and deeper lesions of the intestines may occur.

**Cholera Toxins.**—Koch assumed that the severe symptoms of cholera were due to the absorption of a toxin produced by the growth of the vibrio in the intestines. The toxic effects are apparently due to substances which are an integral part of the organism and are only liberated by the breaking down of the vibrio. Suspensions of killed vibrios, when injected into animals, give the same symptoms as living cultures, although quantitatively less toxic. The endotoxin is labile, and is best shown in cultures which are killed by chloroform or by heating to 56° C. for one hour. More active chemicals or a higher degree of heat changes it from a more specific toxin to a more general protein poison. The bacteria-free filtrates of fresh fluid cultures are only slightly toxic; old cultures, however, due to breaking down of the vibrios may be very toxic. This toxicity is due mostly to substances similar in action to the general class of ptomaines. Kraus has, however, been able to demonstrate, what he considers an extracellular toxin, in young broth cultures. Metchnikoff and Roux have also attempted to prove the existence of an extracellular toxin by growing cholera vibrios in collodion sacs implanted in the peritoneum of guinea-pigs. The production of an antitoxin against such toxins has not been accomplished.

**Communicability.—Origin of Epidemics.**—The two fundamental epidemiological facts are, that the vibrio leaves the body only in the feces, and the mode of infection is by way of the mouth. The feces of the *cholera patient* during the acute stage of the disease are extremely rich in vibrios, which are at times present in almost pure culture. As the case recovers they decrease in number, but persist after recovery for seven to fourteen days, in exceptional instances longer. In only one case have they persisted for three months, that is, chronic carriers do not exist. These persons constitute the "*convalescent carriers.*" In this connection, the mild cases which are undiagnosed or overlooked are important. Another group of persons may act as sources of infection, viz., excrete cholera spirilla in their stools. There are the "*healthy or contact carriers.*" Not all persons who ingest cholera develop the disease. In a number, the vibrio will multiply in the intestine to a limited extent and be excreted in the stools, although no clinical evidences of disease are present. These healthy carriers are important not only as insidious spreaders of infection, but they may be potential cases of cholera. Should their resistance be lowered they may develop the disease.

The transfer of the infectious agent to the mouth may occur in several ways: by personal contact, by fomites, and by contamination of food and water. Where a moderate number of cases are developing in a district having fair sanitary conditions, contact, especially with mild cases and carriers, or indirectly, fomites or infection of food are the sources of infection. As other factors enter, such as the contamination of the soil and privy-vaults with subsequent infection of well and river water and of green vegetables, the cases increase in number. Where a general water supply becomes contaminated an explosive, widespread epidemic follows. It is easy to understand how localized epidemics are kept active, by the development of healthy carriers, who help maintain the contamination of their surroundings; and, given also the climatic conditions, such as heat and moisture, to favor the continued vitality or even multiplication outside of the body, how endemic foci persist. The transfer of infection by fomites, such as body and bed linen or dishes, etc., is only dangerous when direct. Drying quickly lessens the danger. Flies may be a factor by mechanical transfer of the virus.

The susceptibility to infection of different individuals varies and conditions may lower or raise the resistance of the individual. The occurrence of healthy carriers illustrates this. Such carriers may be very numerous. Abel and Clausen, for example, found that 14 of 17 persons belonging to families of 7 cholera patients, had cholera vibrios in their stools. In a group of immigrants who were exposed, we found 10 per cent. were healthy carriers.

The resistance of the individual depends upon his general good health. Gastric and intestinal disorders due to indiscreet eating or drinking, or other causes undoubtedly favor infection or in the case of healthy carriers may cause the development of cholera. At the New York Quarantine Station two such cases promptly developed: one after the administration of a dose of salts, the other after a drinking bout.

**Cholera Immunity.**—Eight to ten days after recovery the serum of cholera patients contains protective substances. If a guinea-pig is injected intraperitoneally with living cholera vibrios, and serum from the patient be given, the pig recovers. Similar protective substances are found in the serum of animals injected with sublethal doses of live vibrios or with killed organisms. The serum is not antitoxic, for although it will protect an animal from a lethal dose of living vibrios, by preventing their multiplication, it has little effect, when a fatal dose of killed organisms or toxic extracts is given. Similarly an animal may be highly immune to the injection of living cultures but on intestinal infection will show no resistance to the poisonous products absorbed.

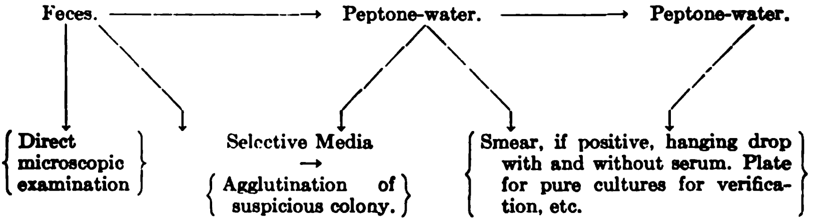
The antibodies present in the serum are precipitins, agglutinins, opsonins, bacteriolytic and bactericidal substances.

**Prophylactic Vaccination and Serum Therapy.**—See Part III.

**Specific Serum Reactions.**—**Agglutinins.**—Because of the acuteness of the disease, the agglutination reaction is valueless for diagnosis. It may be of diagnostic help in recovered cases, where no bacteriological diagnosis has been made. Agglutination is used, however, for identification of the cholera vibrio. In this way cholera and non-cholera vibrios can be separated with certainty, because a cholera-immune serum usually contains few group agglutinins for non-cholera types. Freshly isolated strains agglutinate freely; in fact, seem to give better reactions than stock cultures, although strains vary in their agglutinability; relatively inagglutinable strains such as are encountered among freshly isolated strains of typhoid are not found.

**Pfeiffer Phenomenon.**—This phenomenon (see page 178), which is a bactericidal test *in vivo*, can be employed to differentiate the cholera vibrio from other vibrios. The bactericidal serum should have a high titer, viz., 1 c.c. of a 1 to 1000 dilution should be able to dissolve a loop of cholera vibrios when injected intraperitoneally together. Each factor in the test must be controlled.

## ISOLATION OF CHOLERA VIBRIO FROM STOOLS. OUTLINE OF PROCEDURE.[1]


Feces.
Peptone-water.
Peptone-water.
Direct microscopic examination
Selective Media → Agglutination of suspicious colony.
Smear, if positive, hanging drop with and without serum. Plate for pure cultures for verification, etc.

**Direct Microscopic Examination.**—This is of great value in suspected cases but of no use in the examination of carriers. The presence of many typical, extremely motile vibrios warrants a tentative diagnosis of cholera. In exceptional cases, not cholera, a great abundance of vibrios may be found. This led us in one instance unnecessarily to isolate a nurse, who while caring for a cholera patient, developed nervous diarrhea.

**Peptone-water.**—Inoculate with feces and incubate six to twelve hours. Examine smears from surface growth. If the vibrios are numerous, prepare hanging drops with and without immune serum. If vibrios are not found in the smear, or if too few in number for hanging drop observation, subinoculate into peptone-water, or selective media, or both.

**Subculture Peptone-water.**—This second enrichment is probably never required except in the examination of suspected carriers. In four instances we have found cholera vibrios in subculture where they were not evident in smears from the first peptone culture. This second enrichment helps also to exclude some of the vibrios other than cholera; some will have died out, some will not have enriched beyond the amount present in the first peptone culture; these are not cholera. Smears from the surface growth are examined and if positive, examination of hanging drops carried on. Where haste is not a factor as in carrier examinations the examination of the first peptone culture may be omitted.

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[1] Krumwiede, Pratt and Grund: Jour. Inf. Dis., 1912. x, 134.

In making smears and hanging drops from peptone-water especial care should be taken not to shake the tubes and that the loop is just touched to the surface. In the examination of smears little time need be spent; if very few vibrios are present, further enrichment is necessary. If they are present in sufficient numbers for agglutination, they are found at once.

The use of the surface growth for agglutination is open to certain criticisms, but in practical work it has given us accurate results. Where cholera or cholera-like vibrios, having the same ability to enrich as true cholera, are present, the surface growth is nearly a pure culture. The reliability of the peptone method, as outlined, was best shown in a series of examinations where we found two carriers. In this series 50 per cent. of the stools contained vibrios. The results were checked by the isolation of pure cultures. With some experience a great number of examinations can be carried through in this way, using peptone-water only, with a minimum of preparation and equipment.

**Selective Media.**—Inoculation may be done directly from the feces or after enrichment in peptone-water. The advantage of such media is that they may be heavily inoculated. The colonies which develop are used for agglutination either in hanging drop, or more convenient, the macroscopic slide method (see under Typhoid). Various modifications of Dieudonné's have been suggested. The alkaline-egg medium has the advantage that a distinctive vibrio colony develops allowing quick selection for agglutination should non-vibrios develop. A secondary plating on plain agar is necessary, if pure cultures are to be obtained from selective media. The other bacteria, which are only restrained, may be transferred in fishing a colony and thus yield mixed cultures.

Alkaline agar may be employed for plating either directly from the feces or from peptone enrichments. As stated, the colony is distinctive and no difficulty will be encountered if the vibrios are fairly numerous.[1]

**Saccharose Peptone-water.**—This medium has been suggested by Bendick to avoid the time-consuming microscopic examination of peptone tubes. The stool is first inoculated in peptone-water and the surface growth then inoculated into this medium. Because of the ability of the cholera vibrio to rapidly split saccharose, decolorization occurs in five to eight hours. The tubes which do not decolorize in this time can be discarded, those decolorized are examined for vibrios, which, if present, are isolated by plating.

Because of the presence of a fermentable sugar, the growth is diffused and the surface is not satisfactory for agglutination. To avoid this difficulty duplicate peptone tubes could be planted, however, and used for agglutination when the saccharose peptone tubes were decolorized. This method promises to be of the greatest value where many specimens must be examined in the search for carriers.

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[1] The direct plating of stools on agar or gelatin is sometimes of practical importance. In no disease but cholera do vibrio colonies approach or exceed in number those developing from the ordinary fecal bacteria. Even without identification by agglutination, such a condition gives us a practically certain diagnosis of cholera.

**Examination of Suspected Carriers.**—The simplest procedures possible must be employed when the daily examinations may run into many hundreds or even thousands. The peptone method outlined reduces the bacteriological work very much, especially if the first peptone tubes are not examined. The collection of stools under these circumstances is impracticable. Ordinary swabs moistened in peptone-water may be inserted into the rectum to obtain fecal material. Individual glass specula may be employed to aid in the introduction of the swabs. Where feasible much time is saved by giving an identification card a number and dropping the swab at the time of taking into a similarly numbered tube of peptone-water. The sterilization of the peptone tubes, supported in blocks of wood or racks protected by a cover instead of by cotton plugs, and the use of individual wire loops (sterilized in bundles) for transfers or smears saves a great deal of time. The removal and replacing of cotton plugs and the burning of the platinum loop usually employed is thus avoided. In transferring, the wire is dropped into the second tube. The exposure of the tubes when the cover is removed for inoculation or transfer does not lead to interference by contamination.[1]

**Isolation from Water.**—As the cholera vibrios are few in number in water a large volume should be used. About 1 liter of water is taken and 100 c.c. amounts placed in Ehrlenmeyer flasks and to each is added 10 c.c. of a tenfold strength peptone-water. These are shaken and incubated for eighteen hours. The surface growth is then subinoculated in peptone-water for further enrichment if necessary and plates made for isolation. Especial care must be taken in fishing as vibrios other than cholera may be present in the water, and there is some evidence that cholera vibrios lose their agglutinability to some extent after living in water. These difficulties may be surmounted by many fishings and the use of a high titre serum in low dilutions in order to select cultures for final identification.

**Spirilla (Vibrios) More or Less Allied to the Cholera Spirillum.**—Various types of spirilla may be isolated from stools, water, and other sources. Some are practically identical, morphologically and culturally, with true cholera. It would be well to limit the term "cholera-like vibrios" to this group. Another group of vibrios, similar to cholera in some respects but differing in others might be termed "non-cholera vibrios." Much of the practical interest in these types was lost when the serological methods for identification were introduced. In emergency work, where immune serum is not at hand, they are of extreme practical importance. A few of the types are of interest because of their pathogenicity for animals.

Some of these vibrios enrich in mixed cultures in peptone-water like true cholera, others enrich to a limited extent or die out. The majority of the vibrios found in stools during routine examinations for cholera spirilla can be excluded culturally if serum be not available. Of 50 vibrios thus isolated we found that 43 did not give the cholera-red reaction. Of the 7 that did, 2 produced no acid from glucose and 4 produced acid and gas. One produced acid only, but could be excluded, as it formed a tenacious pellicle on peptone-water.

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[1] Creel: Am. Jour. Public Health, December, 1911.

The following is a short list of vibrios of interest historically or because of their pathogenicity for animals:

*Vibrio metschnikovii;* source, epizoötic gastro-enteritis of fowls, present in intestinal contents and in blood. Typical vibrio with one flagellum, liquefies gelatin and gives cholera-red reaction. A minute amount of culture inoculated into a cutaneous wound causes a fatal vibriosepticemia in pigeons and guinea-pigs.

*Vibrio massavah;* source, stools, considered cholera vibrio when first isolated, four flagella, pathogenicity like Vibrio metschnikovii.

*Vibrio septicus;* source, stools, case of cholerine, cholera-like culturally and morphologically, minute amounts cause a rapidly fatal septicemia in guinea-pigs.

*Spirillum finkler-prior;* source, feces in cholera nostras, does not give cholera-red reaction.

*Vibrio ivanoff* and *Vibrio berlionensis;* source, former artificially inoculated stools for disinfection tests, latter, water artificially inoculated to determine viability of cholera vibrio in water. Both are probably variants produced by artificial conditions; they give the immune reactions of cholera spirilla.

# CHAPTER XXXV.

# PATHOGENIC MICROÖRGANISMS BELONGING TO THE HIGHER BACTERIA (TRICHOMYCETES).

OBSERVERS are still of different opinions in regard to the classification of this group of organisms (see Chapter II).

Foulerton[1] and his associates made an extensive study of this group, both saprophytic and parasitic varieties, and they agree with some others in calling attention to the acid-fast character of some of the varieties and to the apparent relationship of the group to B. tuberculosis, B. mallei, and B. diphtheriæ. To us, however, the relationship does not seem to be close enough to place all of these organisms in one group. We have shown (Chapter II) that the apparent branching in B. diphtheriæ is not a true branching. If these are not classed with the true bacteria, they should either be put in a group by themselves or be classed with the cladothrix group since their apparent branching takes place in a manner similar to that described as occurring in the latter group.

Foulerton considers all organisms in the group classed as higher bacteria as belonging to a single genus, streptothrix, which he places with the hyphomycetes, or mould fungi, because of their growth in branching threads from spore-like bodies. He says that streptothrix and actinomyces are absolutely synonymous terms, and that the majority of pathologists consider them so.

Wright[2] and others do not agree with this view (see below). More minute work, both clinical and experimental, should be done on this group of infections before a classification can be accepted.

**Leptothrix Infections.**—Leptothrix forms are frequently found in the human mouth (*Leptothrix buccalis*), and a few writers have claimed that under certain conditions these may become pathogenic, but since no corroborative work has been done, and very little is known about the group, no opinion can be formed of the worth of these observations.

**Cladothrix Infections.**—The organisms found in the comparatively few cases which have been considered by their observers to be due to cladothrix have not been minutely enough studied to decide definitely as to their true or false branching, the characteristic chosen to separate them from the nocardia; hence it is difficult to separate the two groups, but an attempt should be made, since the difference said to exist between them is an important one from a morphological standpoint. Clinically, however, according to the reports, the cases cited are very similar to those said to be due to nocardia (streptothrix) and to actinomyces.

*Gasten* found in a case of clinically typical actinomycosis, in which abscess cavities were found along the spinal column, not the usual actinomyces in the yellow, granular pus, but a fine mass of filaments. Cultures grew on all the ordinary media, best at incubator temperature, but also at lower temperature on gelatin. The gelatin-stick culture, which was especially characteristic, formed on the surface a whitish button; delicate threads stretched out in all directions from the point of inoculation. On agar and potato rumpled, folded films formed on the surface, with white deposit which contained spores. Animal inoculation gave positive results only in a few cases of intraperitoneal injection of rabbits and guinea-pigs. Purulent nodules were found in the peritoneum. Gasten called the organism *Cladothrix liquefaciens*.

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[1] Lancet, 1910, clxxviii, 551, 626 and 769.

[2] Jour. Exp. Med., 1898, iii, 421; Jour. Med. Res., 1905, viii, 349, and Osler's Modern Medicine, 1907, i, 327.

*Eppinger* found on postmortem examination of a case of chronic cerebral abscess, which was the result of purulent meningitis, in the pus and abscess walls, etc., a delicate fungoid growth which he succeeded in cultivating on various media. On sugar agar it formed yellow, rumpled colonies which finally developed into a skin. On potato it grew rapidly, but the colonies remained small, at first a white granular deposit, which afterward turned red, and on the twentieth day resembled a crystallized almond. It did not grow well on gelatin. In bouillon it formed on the surface a small white granule, which became deeper in the center as it grew and sank to the bottom as a white deposit. The bouillon remained clear.

Microscopically the fungus consisted of fine threads without branches which exhibited distinct motility. No flagella were observed. It was judged to be a cladothrix, to which the name "asteroides" was given by the author. It proved to be quite pathogenic for rabbits and guinea-pigs, and produced an infection called pseudotuberculosis. Mice were not affected by inoculation.

## THE ACTINOMYCES.

The little clumps produced by this group of parasites were first seen by von Langenbeck in 1845, and the organisms were later discovered by Bollinger (1877) in the ox. They were given the name of actinomyces, or ray fungus, by the botanist Harz. They were reported in human beings by Israel in 1878.

The characteristics of the microörganisms, described first by Boström[1] (1890) and then by Wolf and Israel (1891), differed greatly and have led to confusion. Boström's organism grew best aërobically and developed well at room temperature. He noted the intimate relation of the organism to those on fragments of grain, and this led to the finding of similar microörganisms in the outer world on grains, grasses, etc. There is no doubt that some suppurative processes have been due to organisms having these characteristics, but they do not seem to excite true actinomycosis.

Wolf and Israel described a microörganism from two human cases, which differs from that described by Boström, but agrees with the microörganisms obtained by most of the more recent investigators

[1] Beitr. path. Anat., etc., 1890, ix.

from typical actinomycosis. It grew best under anaërobic conditions and did not grow at room temperature. Its growth was much less luxuriant than Boström's microörganism. On the surface of anaërobic agar slant cultures on the third, fourth, and fifth day numerous minute isolated dew-drop-like colonies appeared, the largest pin-head in size. These gradually became larger and formed ball-like, irregularly rounded, elevated nodules varying in size up to that of a millet seed, exceptionally attaining the size of a lentil or larger. As a rule the colonies did not become confluent, and an apparently homogeneous layer of growth was seen to be made up of separate nodules if examined with a lens. In some instances the colonies presented a prominent center with a lobulated margin and appeared as rosettes. A characteristic of the colonies was that they sent into the agar root-like projections. In aërobic agar slant cultures no growth or a slow and a very feeble growth was obtained. In stab cultures the growth was sometimes limited to the lower portion of the line of inoculation or was more vigorous there. In bouillon, after three to five days, growth appeared as small white flakes, partly floating and partly collected at the bottom of the tube. Growth occurred in bouillon under aërobic conditions, but was better under anaërobic conditions. The organisms grew in branching and interlacing filaments, which later tended to break into segments (see Fig. 169). The microörganism in smear preparations from agar cultures appeared chiefly as short homogeneous, usually straight, but also comma-like or bowed rods, whose length and breadth varied. In many cultures short, plump rods predominated, and in others longer, thicker, or thinner individuals were more numerous. The ends of the rods often showed oval or ball-like swellings. Swollen clubs were formed irregularly in the presence of blood or serous fluids.

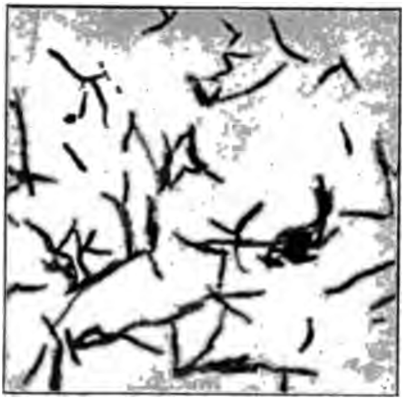

FIG. 169.—Smear from bouillon culture of actinomyces. × 1500 diameters. (From Wright.)

On intraperitoneal inoculation guinea-pigs and rabbits after four to seventeen weeks showed, after being killed, tumor growths mostly in the peritoneal cavity and in one instance in the spleen. Microscopic examination of the tumors showed in all cases but one the presence of typical actinomyces colonies, in most cases with typical "clubs." The general histological appearance of the tumors was like that of actinomycotic tissue.

Wolf in a later paper reports that an animal inoculated in the peritoneal cavity with a culture of the same organism had lived a year and a half. At the autopsy several tumors were found in the peritoneal cavity, and in the liver a large typical tumor in which were many colonies which by microscopic examination were shown to be typical club-bearing actinomyces colonies.

Wright, in 1905, made an extensive study of actinomycosis and added greatly to our knowledge of it.

**Naked-eye Appearance of Colonies of Parasite in Tissues.**—In both man and animals they can be readily seen in the pus from the affected regions as small, white, yellowish or greenish granules of pinhead size (from 0.5 to 2 mm. in diameter). When pus has not formed they lie embedded in the granulation tissue.

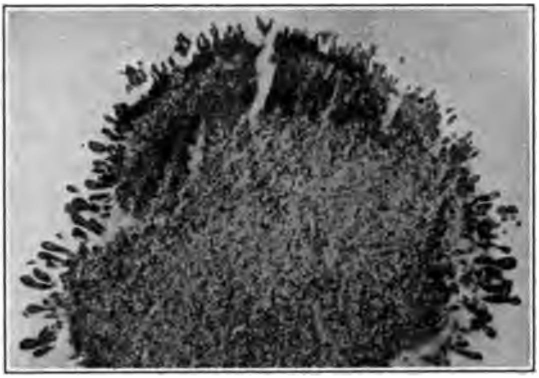

FIG. 170.—A typical "club"-bearing colony of actinomyces. × 325 diameters. (From Wright.)

**Microscopic Appearance.**—Microscopically these bodies are seen to be made up of threads which radiate from a center and present bulbous, club-like terminations (Fig. 170). These club-like terminations are characteristic of the actinomyces. They are generally arranged in pairs, closely crowded together, and are very glistening in appearance. They are more common in bovine than in human lesions. They have been thought to be reproductive elements, but they are probably simply a reaction of the filament end to the host tissue. The threads which compose the central mass of the granules are from $0.3\mu$ to $0.5\mu$ in diameter. The threads show true branching and in the older colonies show a segmentation which gives them the appearance of chains of cocci. Sometimes the whole center of the colonies seems to be a mass of coccus-like bodies most of which are considered spores or conidia; the clubs are from $6\mu$ to $8\mu$ in diameter.

The threads and spores are *stained* with the ordinary anilin colors, also by Gram's solution; when stained with gentian violet and by Gram's method the threads appear more distinct than when stained with methylene blue. The clubs usually lose their stain by Gram's method and take the contrast strain.

**Isolation of Actinomyces.**—Certain strains of actinomyces grow aërobically and others anaërobically. The anaërobic strains are grown with difficulty. A large number of solidified blood serum or serum agar tubes are inoculated with the hope that one or two will develop a growth. The cultures appear much like those of tubercle bacilli. They grow, however, into the medium, and take on a yellowish hue. Wright recommends that granules, preferably obtained from closed lesions, are first thoroughly washed in sterile water or bouillon and then crushed between two sterile glass slides. (In bovine cases make sure the granule has filamentous masses, for, if not, no culture will grow.) The crushed granule is transferred to a tube of melted 1 per cent. glucose agar at 40° C. The material is thoroughly distributed by shaking and the tube placed in the incubator. A number of granules after washing should be placed on the inside of a sterile test-tube and allowed to dry. In this way, should the material be contaminated, the drying of the granules for several weeks may kill off the other organisms. The tube should be examined daily. If a number of living filaments were added to the agar a large number of colonies will develop. These will be most numerous in a zone five to twelve millimeters below the surface (microaërophiles).

The cultures are quite resistant to outside influences; dried, they may be kept for a year or more; they are killed by an exposure of five minutes to a temperature of 75° C.

**Experimental Inoculation in Animals.**—True progressive infection is rarely or never obtained by the injection of pure cultures into rabbits, guinea-pigs, or other small animals. In cattle, however, the disease has been produced from cultures. The cultures form the characteristic "club"-bearing colonies in the tissues of experimental animals. These colonies are either enclosed in small nodules of connective tissue or are contained in suppurative foci within nodular tumors made up of connective tissues in varying stages of development.

Wright does not accept the prevalent belief, based on the work of Boström, Gasperini, and others, that the specific infectious agent of actinomycosis is to be found among certain branching microörganisms, widely disseminated in the outer world. He thinks that these forms belong to a separate genus, *Nocardia*, and that those cases of undoubted infection by them should be called nocardiosis and not actinomycosis. The term actinomycosis should be used only for those inflammatory processes the lesions of which contain the characteristic granules or nodules. That *Nocardia* ever forms these characteristic structures in lesions produced by them has not been convincingly shown.

**Occurrence.**—Actinomycosis is quite prevalent among cattle, in which it occurs endemically; it is more rare among swine and horses. Many cases have in recent years been reported in man. The disease is rarely communicated from one animal to another and no case is known where a direct history of human contagion has been obtained. The cereal grains, which from their nature are capable of penetrating the tissues, have been found in centers of actinomycotic infection in the lower animals. The microörganism may also be introduced by means of carious teeth. Cutaneous infection has been produced by wood splinters, and infection of the lungs by aspiration of fragments of teeth containing the fungus. The presence of the microörganism in cereal grains, which was formerly accepted, is denied by Wright and therefore certainly placed in doubt. The further distribution of the fungus after it is introduced into the tissues is effected partly by its growth and partly by conveyance by means of the lymphatics and leukocytes. Not infrequently a mixed infection with the pyogenic cocci occurs in actinomycosis.

**Characteristics of Disease in Man and Animals.**—In the earliest stages of its growth the parasite gives rise to a small granulation tumor. not unlike that produced by the tubercle bacillus, which contains, in addition to small round cells, epithelial elements and giant cells. After it reaches a certain size there is great proliferation of the surrounding connective tissue, and the growth may, particularly in the jaw, look like, and was long mistaken for, osteosarcoma. Finally, suppuration occurs, which, according to Isreal, may be produced directly by the fungus itself.

The course of the disease is very chronic. Usually the first sign is a point of infiltration about the lower jaw or lower on the neck. This almost painless swelling increases and finally softens in its center. The necrotic tissue finally forces a passage externally or, passing downward, infects the pleura, lungs, mediastinum, or ribs. As a rule the disease is not accompanied by fever. In cattle the disease is usually situated in some portion of the head, especially in the jaw, tongue, or tonsils, hence called lumpy jaw, wooden tongue, etc. Primary lung, intestinal, and skin lesions are not infrequent. These local lesions sometimes scatter and produce a general infection and the udder may be involved.

**Treatment.**—In 1892 Nocard showed that cases in animals might be cured by iodide of potassium, calling attention to the fact that Thomassen had recommended this treatment in 1885. It is given in doses of 1½ to 2½ drams once a day. Salmon and Smith (U. S. Bureau of Animal Industry, Circular No. 96) give directions as to its use.

**Mycetoma (Madura Foot).**—This is a purulent inflammation of the foot occurring primarily in warm climates. The inflammation is accompanied by much irregular enlargement of the foot. Three varieties of this condition have been described based upon the color of the granules found in the diseased area: (1) white, (2) black, and (3) red. The white variety has been studied by Musgrave and Clegg[1] (1907), who have isolated an organism resembling somewhat actinomyces and somewhat the organism isolated by Wright (1898) from a black variety disease. There were repeated attacks of profuse sweating. On the day before her death three indurated swellings beneath the skin were noticed. One, on the left forearm, about the size of a walnut, apparently contained pus. Two, of smaller size, were situated in the right groin.

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[1] Phila. Jour. Sc., 1907, iii, 2, 477.

Blood cultures from a vein in the arm, taken on the sixth day, remained sterile. The leukocyte count on the seventh day was 36,000.

**Autopsy.**—On the right arm, the left forearm, the abdominal wall, and on both thighs there were eight to ten slightly projecting, rounded, fluctuating, subcutaneous swellings from ½ to 1 inch in diameter. The skin over most of these nodules was unaltered, but over the larger ones there was a slight bluish discoloration. The nodules were composed of bluish gray, thick, mucilaginous matter, which was very tenacious and could be drawn out into long threads. The lower lobe was thickly studded with miliary tubercles, and scattered through the entire lung were suppurating foci. Liver and spleen normal. Kidneys: The surface was evenly dotted with minute white spots, which suggested septic emboli rather than tubercles. A few prominent white nodules from ¼ to ½ inch in diameter, contained thick, tenacious matter (Fig. 171). Section showed that the entire substance of the kidney was densely studded with these minute white granules.

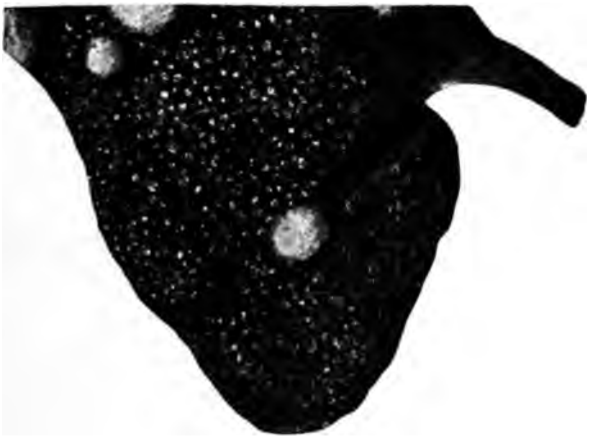

FIG. 171.—Portion of kidney showing minute and large areas of infection.

The gross pathological conditions were interpreted before nocardia was found as follows: An old tuberculous nodule in the right lung; acute miliary tuberculosis in the right lung and peritoneum; acute lobar pneumonia, affecting the left lung; septic infarctions and pyemic abscesses of both lungs, heart muscle, both kidneys, pancreas, mesenteric lymph nodes, and subcutaneous connective tissue. The miliary tubercles of the right lung and peritoneum presented the characteristic appearance of genuine tuberculosis. They were minute, hard, gray, almost translucent nodules, while the granules in the kidneys were of an opaque white or yellowish-white color.

**Microscopic Examination.**—Smears from the abscess beneath the skin and on the surface of the kidneys were stained with methyl blue, carbol-fuchsin, and by Gram's method. The smears resemble those made of tenacious sputum. There is a large amount of mucoid material containing a considerable number of leukocytes. Occasionally irregularly curved, thread-shaped microörganisms are found. They vary considerably in length and thickness, and broken and apparently degenerating fragments are seen. The more slender threads are evenly stained, but some fragmentation or beading of the protoplasm can generally be observed. The thicker threads and broken fragments show deeply stained globules and irregular bodies in a faintly visible rod or thread-shaped covering. Some branching threads are observed, but more commonly they are not branching. No other microörganisms are found in the smears. Sections from the lower lobe of the right lung, stained with hematoxylin and eosin, show in certain places the identical microscopic appearances which are considered characteristic of tuberculosis. Stained by Gram's method, with care not to decolorize too completely, threads like those described in the abscesses are found in great abundance, but rather faintly stained. No threads can be found within the typical tubercles with giant cells, but in the zones of small cells around them they are seen in great numbers, winding about among the cells and forming a sort of network. In the minute foci of small cells one or two fragments of threads are generally seen, and a moderate number in the small abscesses. In the areas of more diffuse infiltration these threads are abundant. No other microörganisms can be found except in the pneumonic area of the left lung, where some groups of cocci are seen.

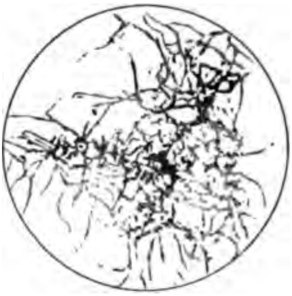

FIG. 172.—Streptothrix from bouillon culture. (From Tuttle.)

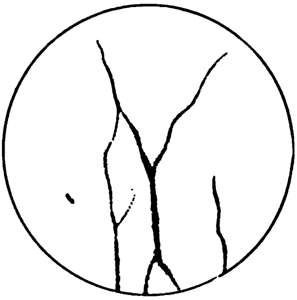

FIG. 173.—Young streptothrix threads showing terminal buds. (From Tuttle.)

**Culture Experiments.**—Six tubes of Löffler's blood serum were inoculated from the kidneys and kept at 37° C. On the third day minute white colonies appeared in some of the tubes, and on the fifth day all the tubes showed from three to ten or twelve similar colonies in each. The colonies increased in size until some of them reached a diameter of one-eighth of an inch. The color, at first white, changed to yellowish-white and then to a decided pale yellow. The well-developed colonies clung firmly to the surface of the medium and were not easily detached or broken up. The growths in all of the tubes were absolutely pure, and consisted of branching threads like those found in the sections.

Löffler's blood serum or other blood medium seems to be the most suitable for cultures.

On plain agar and glycerin agar the growth is the same as on blood serum, but is less rapidly developed.

# CHAPTER XXXVI.

# FILTRABLE VIRUSES. DISEASES OF UNKNOWN ETIOLOGY.

## FILTRABLE VIRUSES.

THERE exists a class of infectious diseases from which it has been quite impossible up to the present time to demonstrate visibly any individual microörganisms, although infective material from such diseases may with certain precautions, be passed through stone-filters of varying degrees of porosity (for types of filters, see Chapter IV); the filtrates will contain the virus and be capable of reproducing the disease with all its characteristics when inoculated into a susceptible animal. Examined microscopically, even with the highest powers, the filtrate is limpid, and, except in a few diseases which will be described in detail later on, not a sign of characteristic particulate matter can be seen. Such a filtrate therefore contains either ultramicroscopic organisms or small organisms with refraction and staining powers so faint that they cannot be demonstrated by our present methods.

Certain precautions must be observed in such filtrations. In the first place the filter must be shown by actual test to be free from infection. Any and all of the test organisms must be absolutely retained and none pass into the filtrate. A few minute species of germs have been found to pass certain grades of filters, especially under pressure. Thus the bacillus of guinea-pig pneumonia, which is $0.5\mu$ x $0.7\mu$, passes Berkefeld V (Wherry); a spirillum isolated by von Esmarch passes, according to him, the Berkefelds, Chamberland F, and other filters; a minute water flagellate was found by Borrell to pass through the coarser filters. Wolbach and Binger and others have shown that several spirochetes, pathogens as well as saprophytes, pass through Berkefelds V, N, and W, by pressure and by suction. It is seen, therefore, that certain known organisms within the limits of visibility may pass even some of the finer filters. The filtration must be completed within a moderate time, because even bacteria as large as the typhoid bacillus may, in media containing a certain amount of albuminous material, grow, in time, through the filter. The material to be filtered should, furthermore, be greatly diluted and first filtered through filter paper or other coarse filter in order to avoid the clogging action of extraneous material.

If after all the proper precautions have been taken the filtrate is pathogenic, it must be shown that the symptoms are due to a microorganism and not to a toxin. This may be decided by inoculating a series of animals successively with the filtrate obtained from a previously so inoculated animal.

From our present knowledge, filtrable agents may be divided into two groups: (1) those which have not yet been morphologically demonstrated (ultramicroscopic?); (2) those which are shown to be within the limits of visibility. A third group may be made of the diseases produced by viruses of questionable filtrability.

## GROUP I.—DISEASES PRODUCED BY FILTRABLE AGENTS OF UNKNOWN MORPHOLOGY.

**Foot-and-mouth Disease.**—This is probably the first disease shown to be produced by a filtrable agent. It is a highly infectious disease of cattle. Other domestic animals may also be attacked. It occurs seldom in man and then among people handling infected cattle or drinking the milk of infected animals. The disease in cattle is characterized by the appearance of vesicles in the mouth and around the coronet of the foot as well as between the toes.[1] Löffler and Frosch[2] in 1898, discovered that after diluting the contents of an unbroken vesicle with twenty to forty times its volume of water and passing the resulting fluid through a Berkefeld filter, the filtrate contains the virus. This virus remains infectious for some time. One attack of the disease usually produces a certain amount of immunity. Löffler claimed to have produced a serum which has immunizing properties but his work has not been corroborated.

**Mosaic Disease of Tobacco.**—This is the second disease in which a filtrable virus was demonstrated. The disease causes the young tobacco leaves to become devoid of chlorophyll in spots which enlarge, turn brown, and the underlying tissue becomes necrotic. Beijerinck, in 1899, showed that the filtrate from a porcelain filter promptly reproduced the disease on tobacco leaves.

**Cattle Plague (Rinderpest).**—This fatal European and African disease of cattle is characterized by inflammation of the intestinal mucous membrane. The blood is infectious and filtrates of it through Berkefelds and Chamberland F (Nicolle and Adil-Bey, 1902) produce the disease. No organism can be seen. Immunity follows one attack. It can also be produced by a subcutaneous inoculation of bile from an infected animal.

**Yellow Fever.**—The undiluted serum from cases of this disease has been shown by the American commission (1901) and others (see p. 633) to pass the Berkefelds and the Chamberland B filters as clear filtrates, and in this form to be infectious; therefore some forms at least of the specific organism are probably ultramicroscopic.

**Rabies.**—No bacteria have been discovered that are considered as factors. The probability of the Negri bodies being protozoa and the exciting factor is considered in Chapter XLV. The virus of rabies has been shown to be filtrable (Remlinger, 1903). Poor and Steinhardt[3]

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[1] Mohler: Farmers' Bull., 666, U. S. Dept. Agric., 1915.

[2] Centralbl. f. Bakt., 1898, xxiii, 377.

[3] Jour. Inf. Dis., 1913, xii, 202.

(1913) showed that gland virus is more filtrable than that from the brain. It may pass through the coarser Chamberland filters.

**Trench Fever.**—Soon after the beginning of the great war there began to appear among the troops in France, especially those in trenches, many cases of acute fever characterized by dizziness, headache, backache and pains in the legs. The temperature curve shows an intermission on the third or fourth day, followed after a day or two by a second rise and a fall about the eighth day from beginning. There may be from one to several relapses at fairly regular intervals. There is no mortality. Many studies have been made to show that the disease is an infectious entity. McNee, Renshaw and Brunt[1] proved that the whole blood could transmit the disease to healthy soldiers by intravenous or intramuscular inoculation. A few experiments by Davies and Wildon[2] showed that lice probably transmitted the disease. This was proved by members of the Medical Research Committee of the American Red Cross headed by Strong.[3] They have summed up their work as follows:

1. Trench fever is a specific infectious disease.
2. The organism causing the disease is a resistant filtrable virus.
3. The virus causing trench fever is present particularly in the plasma of the blood.
4. The disease is transmitted naturally by the louse Pediculus humanus, Linn., var. corpus, and this is the important and common means of transmission. The louse may transmit the disease by its bite alone, the usual manner of infection, or the disease may be produced artificially by scarifying the skin and rubbing in a small amount of the infected louse excrement.
5. A man may be entirely free from lice at the time he develops trench fever, the louse that infected him having left him some time previously, and the louse need only remain upon the individual for a short period of time in order to infect him.
6. The virus of trench fever is sometimes present in urine and sputum.
7. Since urine and sputum are sometimes infective they should be sterilized.
8. To prevent trench fever, or limit its spread and thus save manpower greater efforts should be made to check infestation with lice.

Spirochetes and several kinds of minute organisms have been respectively described as the cause of trench fever but further studies are awaited for proof.

**Hog Cholera.**—de Schweinitz, Dorset, Bolton, and McBryde demonstrated in 1905 that the blood of hogs suffering from hog cholera contains a filtrable virus which is capable of producing the disease on inoculation into healthy hogs. This virus passes Chamberland B and F filters. It leaves the body in the urine and probably enters another animal through contaminated food. King, Baeslack, and Hoffman[4] found in 1913 a short motile spirochete (Spirocheta suis) in the blood in a series of cases of hog cholera. They think this organism may be a stage of the specific organism which may produce filtrable granules.

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[1] British Med. Jour., 1916, i, 225.

[2] Jour. Roy. Army Med. Corps, 1918, xxx, 92.

[3] Trench Fever Report of Commission Medical Research Committee, American Red. Cross, 1918, University Press.

[4] Jour. Inf. Dis., 1913, xii, 39 and 206.

This disease is a very fatal and contagious disease of swine characterized by fever and ulcerative enteritis. Immunity follows one attack. An immune serum is produced. Animals are also immunized by a mixture of infected blood and immune serum (sensitized virus). The hog cholera bacillus was earlier supposed to be the cause of this disease.

**South African Horse Sickness.**—This is a warm-weather disease, more common in animals that do not pass the night under cover. The horses are uneasy, have difficulty in breathing, and a reddish froth exudes from their mouths. The temperature rises in the daytime, but has a tendency to drop at night. In severe cases an edematous swelling of the head and neck may appear. MacFadyen succeeded in passing blood serum of a diseased horse (diluted) through the Berkefelds and a Chamberland F, not through a Chamberland B filter.

**Chicken Sarcoma.**—In 1910 Rous[1] discovered a spindle-celled tumor in chickens, which he has reproduced in other chickens by transplantation, by inoculation of dried tumor tissue, and by the inoculation of Berkefeld filtrates from emulsions of tumor cells.

**Novy's Rat Virus.**—Novy has found an extremely virulent filtrable virus from a disease of rats, which passes all filters. He has not yet published printed reports.

## GROUP II.—DISEASES PROBABLY PRODUCED BY FILTRABLE AGENTS SHOWN TO BE VISIBLE.

**Contagious Pleuropneumonia of Cattle.**—This malady affects cattle but not other species. Typically, there is an inflammation of the lungs and the pleura which is invasive and causes necrosis of the diseased parts. Nocard and Roux succeeded in cultivating the organism in collodion sacs placed in the peritoneal cavity of rabbits, using a mixture of serum and bouillon. After two weeks a very faint turbidity appeared in the sacs; coincidently the fluid became infective. Nocard and Roux described the organisms as minute spheres and spirals just within the limits of visibility. They showed (1899) that the organisms passed the Berkefelds and a Chamberland F, but not a Chamberland B filter. Immunity is produced by a single attack. It has also been produced artificially by the inoculation of cultures or virulent exudates.

**Epidemic Poliomyelitis.**—This is a disease which affects chiefly the central nervous system. It occurs usually in children, and appears sporadically and in epidemics in many countries. In New York, in 1907, there was an epidemic of over 2000 cases and in Texas, in 1912 there was a large outbreak. During the summer of 1916 one of the largest epidemics known occurred in New York City and the surrounding country.[1] In nature, the virus enters probably through the upper air passages. The chief symptoms of the disease are fever, with or without sore throat, hypersensitiveness followed by paresis and paralysis. The mortality is low. There is usually permanent injury to parts of the motor areas of the nervous system, with resulting deformity. The principal microscopic changes are a marked exudation of polynuclear leukocytes into the lymph spaces and the cerebrospinal fluid. The changes are usually specially marked in the anterior commissure and the cornea of the cervical and lumbar regions of the cord, but the whole nervous system is more or less affected. The bloodvessels may become thrombosed and ruptured. The disease seems to be closely related to, if not identical, with certain diseases manifested by acute encephalitis and bulbar paralysis, *e. g.*, Landry's paralysis. That the disease also bears a marked resemblance to rabies is quite apparent. The finding of the virus in the salivary glands and the demonstration of its filtrability give added evidence of its similarity to rabies.

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[1] Rous and Murphy: Jour. Exp. Med., 1912, xv, 270.

Until recently nothing was definitely known of the etiology. Now we have a series of studies which seem to have thrown much light on the subject. Landsteiner and Popper (1909) reported the transmission of acute poliomyelitis to apes. They inoculated the spinal cord intraperitoneally and produced typical symptoms and lesions, but did not succeed in transmitting from ape to ape, probably because they used a mild virus. Flexner (1909) and Lewis[2] transmitted the disease from monkey to monkey by means of intracerebral inoculations. Landsteiner and Levaditi[3] (1909) also transmitted it from monkey to monkey. They found that the virus lives some time outside of the body; that the degenerated nerve cells are taken up by phagocytes, and there is an analogy between the lesions of poliomyelitis and those produced by rabies. They demonstrated that the virus is filtrable. Leiner and Wiesner transmitted the disease from monkey to monkey, found young animals more susceptible than older ones, and spinal fluids, blood, and spleen negative. Flexner transmitted the disease by inoculating into the blood or peritoneal cavity, also by the subcutaneous method, and independently found the virus to be filtrable. Landsteiner and Levaditi found the virus in the salivary glands and suggested the saliva, moist or dry, as a source of contagion.

Soon after this (1911) Noguchi and Flexner[4] announced that they had obtained cultures in media similar to that used by Noguchi in cultivating spirochetes (see p. 509). In such media in about five days the pieces of tissue employed become surrounded by an opalescent haze which increases for about five days more, and a sediment gradually forms. Giemsa's stain shows the presence of minute globoid bodies (0.15 to 0.3 diam.) in pairs, short chains, and masses. Cultures were also obtained from the filtered virus. Monkeys inoculated with certain strains of this organism grown for a variable number of culture generations may die with typical lesions of the disease. The authors consider these bodies the cause of the disease. They further report that the cultures are filtrable. Levaditi states that he cannot obtain the results of Noguchi, but that he obtains evidence of growth by the living tissue method. Rosenow[1] states that he found an aërobic streptococcus which shows in early culture generations specific pathogenicity for the central nervous system, producing paralysis in animals. When grown under anaërobic conditions he says it shows only minute forms. He therefore concludes that it is the cause of the disease and that it is probably the same organism as that described by Flexner and Noguchi. Mather found a similar coccus.

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[1] Monograph Series No. 16, New York City Health Department, 1916; Public Health Bull. No. 91, U. S. Pub. Health Service, 1918.

[2] Jour. Am. Med. Assn., 1909, liii, 1913 and 2095, xli, 1639; Jour. Exp. Med., 1910, xii, 227.

[3] Compt. rend. Soc. de biol., 1909, xlvii, 592 and 787.

[4] Jour. Exp. Med., 1913, xviii, 461.

During this epidemic we also studied the etiology of this disease, but our studies so far have led us to no definite conclusions. We isolated the Flexner-Noguchi organism in 2 cases out of 50, and definite streptococci in more than half the cases. We can say that the Flexner-Noguchi organism is distinctly different from any aërobic streptococcus studied and that it is filtrable, but we have not been able to produce the disease in monkeys with our cultures.

Rabbits and possibly guinea-pigs have also been found to be somewhat susceptible to the virus (Krause and Meinicke,[2] Marks,[3] Rosenau[4]) These authors state that the disease in rabbits does not resemble that in man.

**Immunity.**—One attack seems to give certain immunity. Flexner and Amoss[5] state that, experimentally, the virus inoculated into the blood is capable of being neutralized by intraspinous injections of immune serum. A series of vaccinations similar to that given as a prophylactic against rabies may produce immunity.[6]

Immune reactions with the cocci isolated by Rosenow[7] and by Mathers[8] have been reported by the respective authors.

**Encephalitis Lethargica.**[9]—During 1918 a series of cases occurred in England and France which were first thought by some to be atypical cases of poliomyelitis. That doubt prevailed as to their nature was shown by the various names applied to the condition—acute encephalitis, infective ophthalmoplegia, poliomyelitis, botulism, lethargic encephalitis, etc. The majority of observers now think that the disease is a clinical entity similar to the one described by Enconomo in Vienna in 1917, and to cases described as following the epidemic of influenza in 1890 especially in northern Italy, where it received the name of Nona. The disease is characterized by three cardinal signs: lethargy, asthenia and cranial nerve palsies. Bradford, Bashford and Wilson[10] report finding a filtrable organism similar to the one Noguchi found in poliomyelitis, which when inoculated into monkeys produce symptoms similar to that in man.

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[1] Jour. Med. Res., 1917, xxxvi, 175, and Jour. Inf. Dis., 1918, xxii, 281.

[2] Deutsch. med. Wchnschr., 1909, xxxv, 1825.

[3] Jour. Exp. Med., 1911, xiv, 116.

[4] Ibid., 1916, xxiii, 461.

[5] Ibid., 1913 and 1914.

[6] Abramson: Jour. Am. Med. Assn., 1918, lxx, 1142.

[7] Jour. Am. Med. Assn., 1918, lxxi, 433, and Jour. Inf. Dis., 198, xxii, 379.

[8] Mathers and Harell: Jour. Inf. Dis., 1917.

[9] Neal: Monthly Bull. Health Dept., New York City, 1919, ix, 69.

[10] British Med. Jour., May 17, 1919.

**Relapsing Fever.**—Todd and Wolbach have found (1914) that Spirocheta duttoni may be forced through even the finer grades of Berkefeld filters by a pressure of from fifty to ninety pounds to the square inch. For filtrability of other spirochetes see page 570.

**Mumps.**—Comparatively few studies have been made of this infectious disease, probably because of its low mortality. By puncturing the parotid gland, Laveran and Catrin obtained a diplococcus which stains easily, is Gram-negative, and grows on ordinary culture media. No satisfactory specific studies have been made of this organism. Wollstein[1] showed the virus is filtrable, that half grown female cats react specifically to the virus and that the virus is detected most readily during the first three days of the disease and not on the ninth day.

Hess,[2] in 1915, used serum from recovered cases of mumps in a children's homes as a protective measure, with the result that none of the 17 so inoculated came down with the disease on exposure.

## GROUP III.—DISEASES PRODUCED BY VIRUSES OF QUESTIONABLE FILTRABILITY.

**Smallpox and Related Diseases.**—The details of the disease smallpox are considered in a later chapter. In 1908 Casagrandi reported that the virus was filtrable under pressure through coarser Berkefeld filters. Vaccine virus had already been reported as filtrable by some investigators, and as non-filtrable by others. Our results agree with those of the latter observers. The opinion as to the filtrability of the other "pox" diseases is not unanimous.

**Dengue.**—Ashburn and Craig[3] claim to have reproduced dengue in susceptible individuals by a procedure similar to that employed in yellow fever. The virus they say passes a Berkefeld filter. The intermediary host in natural infection is claimed by them to be Culex fatigans. Lane reports that an epidemic occurring at St. Thomas was apparently due to "Stegomyia calopus."

**Measles.**—This very definite infectious exanthematous disease still remains among those of unknown etiology. Both cell inclusions (Field,[4] Ewing,[5] and others), bacilli and cocci have been reported as having an etiological relationship. The reports have not been corroborated. In 1905 Hektoen[6] produced measles in two human cases by the inoculation of blood drawn from an infected case at an early stage of the disease. Anderson and Goldberger,[7] in 1911, inoculated monkeys with measles blood and demonstrated the virus in the blood of the inoculated animals and in the secretion of the upper air passages. They also demonstrated the period of infectivity of the blood. They reported positive results with Berkefeld filtrates. Sellards and Wentworth[1] report that they were unable to see any reaction in 3 monkeys (M. rhesus) inoculated intraperitoneally with the blood from 5 measles patients, taken within twenty-four hours after the appearance of the rash. A human volunteer inoculated with the blood from these monkeys showed no reaction. No human controls were used for the original human blood. Tunnicliff[2] has reported finding a small anaërobic coccus in the blood of 42 out of 50 cases of measles. She states that there seems to be a specific increase of opsonins, complement-fixing bodies and agglutinins for this coccus early in measles patients. Hektoen[3] gives a review of the bacteriology of measles.

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[1] Jour. Exp. Med., 1916, xxiii, 353, and 1918, xxviii, 377.

[2] Am. Jour. Dis. Child., 1915, x, 99.

[3] Jour. Inf. Dis., 1907, iv, 440.

[4] Jour. Exp. Med., 1903, vii, 343.

[5] Jour. Inf. Dis., 1909, vi, 1.

[6] Ibid., 1905, ii, 238.

[7] Public Health Reports, 1911, 1912 and 1913; Jour. Am. Med. Assn., 1911, lvi, 113.

**Rubella.**—Tunnicliff[4] has also found a coccus in a series of rubella cases for which she has reported specific antibodies.

**Scarlet Fever.**—Scarlet fever is an acute febrile, highly infectious disease, characterized by a diffuse, punctate, erythematous skin eruption, accompanied by catarrhal, croupous, or gangrenous inflammation of the upper respiratory tract and by manifestations of systemic infection. The disease was probably known long before the Christian era, but the present name does not appear until the time of Sydenham (1685), who differentiated the disease from measles. It is very generally disseminated, but is much more common in temperate climates than in the tropics. The specific exciting factor is thought by some to be a streptococcus, of the Streptococcus pyogenes type, but the evidence in favor of this view is very slight (see Streptococcus pyogenes).

Mallory[5] (1914) found certain bodies occurring in a series of forms in and between the epithelial cells of the epidermis and free in the superficial lymph vessels and spaces of the corium. He gave the name Cyclasterion scarlatinale to these bodies in consequence of the frequent wheel and star shapes of the rosette. Field and others decided that these bodies are not specific germs. Mallory reports the finding of a small pleomorphic bacillus which he thinks may be the specific cause. The reports that a filtrable virus is found lack corroboration.

**Trachoma.**—This condition has already been considered in a previous chapter. Bertarelli reported that he was able to produce a specific filtrate. Nicolle[6] and Noguchi[3] also reported positive results with filtrates. The descriptions of the disease, however, are too vague to allow an opinion as to the truth of these reports.

**Typhus Fever.**—The occurrence of this infectious disease in epidemic form had disappeared from civilized lands until the recent war broke out. The fact that the body louse may transmit the disease helps explain why it has always been classed as a filth disease. It still occurs endemically in parts of Europe and North and South America. In Mexico it occurs in epidemics where it is known as Tabardillo. In New York it occurs occasionally in mild form under the name of Brill's disease. Brill thought it was a new disease, but Anderson and Goldberger[1] have shown that typhus fever and Brill's disease are the same. Nicolle and Krumwiede[2] observed four cases which clinically verified these findings. The disease is characterized by high temperature and a petechial rash.

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[1] Johns Hopkins Hosp. Bull., 1919, xxx, 57.

[2] Jour. Am. Med. Assn., 1917, lxviii, 1029.

[3] Ibid., 1918, lxxi, 1201.

[4] Jour. Inf. Dis., 1918, xxii, 462.

[5] Mallory and Medlar: Jour. Med. Res., 1916, xxxv, 209.

[6] Compt. rend. Acad. Sc., 1909 and 1911; Ann. de l'Inst. Past., 1910, 1911 and 1912.

Nicolle (1909) showed that the old world typhus can be transmitted to the chimpanzee and from this to the macacus with typical eruption in each case. He also showed that the disease is transmitted by the ordinary body louse (Pediculus vestimenti), seven days after an infected meal, and not after the tenth day. Anderson and Goldberger (1909) were the first to transmit typhus fever of Mexico (tabardillo) to monkeys. They were able to transmit directly from human beings to the macacus and capuchin and from monkey to monkey. Ricketts and Walker (1910) also found that the macacus was directly susceptible to the disease. They based their diagnosis chiefly upon a rather indefinite fever and in most cases somewhat distinct symptoms of illness. They also found that the monkey may pass through an attack of typhus so mild that it cannot be recognized clinically, but it results in immunity. The immunity test is a reliable proof of the previous occurrence or non-occurrence of typhus at least within a period of one month. They found that typhus was transmitted to the monkey by the bite of the louse. They further state that in stained preparations of blood of patients taken from the seventh to the twelfth days of the disease they invariably found a few short bacilli similar to those which belonged to the hemorrhagic septicemia group. In moist preparations they saw similar forms in all cases. No motility was observed. No cultures could be obtained. They examined the dejecta of many lice and found similar bodies in large numbers in infected lice and occasionally in non-infected lice. Pediculus capitis may also transmit the disease.

Plotz and co-workers[3] reported the isolation in pure cultures of a Gram-positive pleomorphic anaërobic bacillus from cases of typhus and of Brill's disease. Their report is given on page 503. The claim that this bacillus is specific lacks corroboration.

First attempts at filtration by Ricketts and Wilder,[4] Anderson and Goldberger[5] and Nicolle,[6] Connor and Wilder gave negative results. Olitzky[7] went over this work and added experiments of his own which seemed to show that the virus is non-filtrable. Nicolle and co-workers have later reported some positive results.

*Agglutinins in Blood of Typhus Fever Patients.—Weil-Felix Reaction.*—Wilson[8] called attention to the presence in the serum of typhus fever patients of agglutinins for intestinal colon-like bacilli. The so-called

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[1] Public Health Rep., 1910, 1912 and 1913; Jour. Am. Med. Assn., 1911, lvii, 113.

[2] Jour. Am. Med. Assn., 1912, lix, 521.

[3] Ibid., 1914, lxii, 1556.

[4] Ibid., 1910, lx, 309.

[5] Public Health Rep., 1910, 1912 and 1913; Jour. Am. Med. Assn., 1911, lvii, 173.

[6] Compt. rend. Acad. Sc., 1909 and 1911; Ann. de l'Inst. Past., 1910, 1911 and 1912.

[7] Jour. Inf. Dis., 1917, xx, 349.

[8] Jour. Hygiene, 1909, ix, 332 and 1910, x, 155.

Weil-Felix[1] reaction takes advantage of this fact. They employ a strain of B. proteus called X 19 and have found that a positive agglutination in dilutions over 1 to 50 (macroscopic method) can be considered diagnostic. The reaction is usually positive by the sixth day. The agglutinin content usually continues to rise, so that titers of 1 to 1000 or more are common. The test should be repeated, if doubtful or if there is an associated positive Widal reaction.

**Other Diseases said to be due to Filtrable Viruses.**—Several other diseases of less importance have been listed as belonging to those produced by a filtrable agent. When a well-known disease such as epidemic cerebrospinal meningitis is reported as being due to a filtrable virus, much corroboration is needed before we can accept the statement as a fact.

Kruse[2] corroborated by Foster[3] reported that a filtrable virus is obtained from common colds, which would reproduce the disease.

The recent reports that came from two groups of English observers[4] claiming that the etiologic agent in a whole group of diseases is a minute microörganism specific for each disease, similar to Noguchi's poliomyelitis coccus, have been discredited.[5]

## OTHER DISEASES OF UNDETERMINED ETIOLOGY.

**Rocky Mountain Spotted Fever.**—This is an acute infectious disease characterized by fever and a more or less hemorrhagic eruption. Some years ago Wilson and Chowning thought they found a protozoan in the blood similar to babesia in Texas fever. Their findings have not been corroborated. Their investigations proved, however, that rabbits are susceptible to the disease and that a tick of the genus Dermacentor probably carries the infection. Then Ricketts and Gomez made some very interesting studies on the disease. They found that guinea-pigs and monkeys are susceptible as well as rabbits, and they further found that in guinea-pigs and monkeys an attack of spotted fever produces a strong active inherited immunity characterized by a serum with high-protective but low curative power, and that the production of the serum in the horse with the use of serovaccination in man may give practical results. They found a moderate number of diplococcoid bodies in the blood of infected guinea-pigs and monkeys, and fewer in man. They found that the virus is transmitted by the infected female tick (wood-tick—Dermacentor Venustus) to her young through the eggs. If the larvæ from these eggs are allowed to feed upon normal guinea-pigs, these animals come down with the disease. Immense numbers of these apparent organisms are found in affected eggs and none were found at first in normal eggs. Afterward Ricketts found a few, but he thought these might be an avirulent species of the same organism. The salivary glands, alimentary sac and ovaries of infected female ticks are swarming with these bodies, while normal ticks seem to have none. Lastly, Ricketts found that these bodies agglutinate with specific serum, 1 to 300 dilution. Wolbach[1] has corroborated some of these findings and demonstrated a similar organism in characteristic lesions of experimental animals bitten by infected ticks. Frick[2] recently reported the finding of small bodies in centrifuged blood cells, which he did not classify.

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[1] Wien. klin. Wchnschr., 1918, p. 11. Craig and Fairley: Lancet, 1918, ii, 385.

[2] Münch. Med. Wchnschr., 1914, lxi, 1547.

[3] Jour. Am. Med. Assn., 1916, lxvi, 1180.

[4] Gibson, Bowman and Conner: British Med. Jour., December 14, 1918 and March 22, 1919. Bradford, Bashford and Wilson: Lancet, February 1, 1919, and British Med. Jour., May 17, 1919.

[5] Arkwright: Brit. Med. Jour., August 23, 1919, p. 233.

Cumming[3] studied 2 cases occurring in California and reported a reaction in monkeys and an apparently specific swelling of the scrotum in a series of guinea-pigs from the blood of the original monkey.

**Chicken-pox.**—Kling[4] claims to have been able to vaccinate against this contagious disease with the clear contents of a fresh vesicle in as early a stage as possible. He inserted the point of a sterile lancet into such a vesicle and then into the patient's skin, repeating six times. This work was corroborated by Rabinoff.[5] Hess and Unger[6] report immunity by a similar method.

**Pellagra.**—This disease has been much studied recently. The theory that it is due to the ingestion of damaged corn received a check from the work of Siler, Garrison and MacNeal (1913) who stated that they were unable to get evidence to support this theory. They consider the disease a specific infection caused by unknown means. Since then the work of Goldberger and others added much to the evidence that the disease is due to a deficiency of certain substances in the diet which may be corrected by including in the diet suitable proportions of fresh animal and leguminous protein food.

**Verruga peruviana,** a South American disease has been shown by Strong and his co-workers[7] to be a disease distinct from oroya fever (see p. 606) and to be produced by an unknown virus transmitted, according to Townsend[8] by the bite of a gnat (Phlebotomus verrucanamun).

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[1] Jour. Med. Res., 1912, xxviii, 1 (with bibliography); also 1916, xxxiv, 121 and xxxv, 147.

[2] Public Health Rep., March 3, 1916, p, 516.

[3] Jour. Inf. Dis., 1917, xxi, 509.

[4] Hygiene, 1913, lxxv, 1032; Berl. klin. Wchnschr., 1915, lii, 13.

[5] Arch. of Ped., 1915, xxxii, 651.

[6] Am. Jour. Dis. Chil., 1918, xvi, 34.

[7] Jour. Am. Med. Assn., 1913, lxi, 1713.

[8] Ibid., 1913, lxi, 1717.

# CHAPTER XXXVII.

# PATHOGENIC PROTOZOA.

THE classification and general characteristics of protozoa are given in Part I. The individual groups and pathogenic members will now be described. With the intermingling of troops from the whole world during the recent war the possibility of increased infections[1] from known pathogenic protozoa and of evidence of pathogenicity among doubtfully pathogenic genera must be considered.

## FLAGELLATA.[2]

The flagellates that are distinctly pathogenic to man belong chiefly to the genera Trypanosoma and Leishmania, but certain other genera are found occasionally in man which may be mentioned because of the possibility of their becoming pathogenic. According to the classification of Calkins (1908) the flagellates parasitic in man are from three orders, the Monadida, the Heteromastigida, and the Polymastigida; according to that of Doflein and Koehler[3] (1913), they are from two, Protomonadina (one to three flagella), and Polymastigina (four to eight flagella). Six families, according to the morphology of the flagella, are distinguished, three of which contain forms parasitic for man, namely, Cercomonadidæ (one flagellum and no blepharoplast), Trypanosomedæ (one flagellum, a blepharoplast, and an undulating membrane), and Bodonidæ (two flagella).

Under the Trypanosomedæ, which include most of the pathogenic forms, are placed the genera Leptomonas, Herpetomonas, Leishmania, Trypanosoma, and Schizotrypanum and Endotrypanum. Under the Bodonidæ are placed the genera Bodo and Prowazekia, and in the order Polymastigina the genera Trichomonas and Lamblia have been found in human beings.

Hartmann puts the Trypanosomata, with other blood parasites, in an order, the Binucleata, and makes the Spirocheta an appendix of this order. According to this arrangement the Hemosporidia are taken from the Sporozoa and placed in the Binucleata with the Trypanosomata. The malarial organisms supposedly lose through their parasitism many of the characteristics ascribed to this order.

**Material and Methods for Study.**—A number of flagellates (Bodo, for instance, see p. 572) are found in the large intestine of the lower animals. The toad, the grass lizard, and the guinea-pig may contain some interesting forms. As these forms are easily obtained and remain alive a long time outside of the body, they are well fitted for class study.

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[1] Kofoid, Kornhauser and Plate: Jour. Am. Med. Assn., 1919, lxxii, 1721.

[2] See Part I for general description of Protozoa.

[3] Kolle and Wassermann, 2d ed., 1913.

The feces are obtained by pressing lightly over the anus of the animal, or if the whole intestinal tract is to be examined, by sacrificing the animal and dissecting out the parts wanted. The material is placed in a clean watch-glass and thinned if necessary with physiological salt solution. Hanging drops may be made in physiological salt solution or in such a solution made a little thick by the addition of gelatin in order to retard the motion of the flagellates somewhat so they may be better studied.

Permanent preparations may be made according to directions given in Part I. As most of the pathogenic members of this group may be difficult to obtain in the living condition at any stated time, they must be studied by students principally in stained smears and sections.

If one can obtain rats infected with Tr. lewisi, others with one or more pathogenic forms, still others with Spirocheta obermeieri, the infecting organisms can be kept alive by frequent reinoculation of the heart's blood, subcutaneously or intraperitoneally into the fresh animal, or cultures may be carried on (see below). But this is an expensive and tiresome work in those laboratories where such work is not being carried on, and generally one must rely on the permanent preparation. In the development in the second host one must also study the stained specimens in the great majority of instances.

The fresh specimens of blood are obtained from the tail tip of the rat, or the ear of the dog; they may be examined, after dilution with physiological salt solution, in the hanging drop, or in a drop spread under a cover-glass and ringed with vaselin. For permanent preparations films of the blood are spread, fixed, and stained in the usual way; Giemsa's method of staining (see stains) is very satisfactory.

For section work of the various organs the fixatives and methods given in Chapter III may be used. Special methods are given under each organism.

**Artificial Cultures of Blood Flagellates** (see Chapter IV).—These, according to Novy and MacNeal,[1] may be made on a culture medium consisting of a mixture of ordinary nutrient agar with variable amounts of fresh defibrinated rabbit or rat blood. The best all-around results are obtained with equal parts of blood and agar. The agar is melted and cooled to 50° C., then the blood is added and thoroughly mixed. The tubes are inclined until the medium stiffens, when they should be inoculated at once with blood or other infected material containing living trypanosomes. The surface of the medium should be very moist, so water of condensation may form. Generally evidence of growth may be observed in three or four days.

## CERCOMONAS.

The members of this genus are round or oval flagellates with a long anterior flagellum and a more or less pointed posterior extremity which is sometimes ameboid. The vesicular nucleus is situated anteriorly, and passing through the organisms from flagellum to posterior extremity is an axial elastic fibril (Fig. 172, *a*). Division into two daughter forms has been observed.

A number of cercomonada, none of them well studied, have been observed in different animals as well as in man. They are very numerous in stagnant water.

*Cercomonas hominis* (Davaine, 1854) was observed in the dejections of a cholera patient by Davaine. The body is $10\mu$ to $12\mu$ long and pear-shaped, pointed posteriorly. The flagellum is twice as long as the body. Davaine also reported a smaller form in the stools of a typhoid patient. Other observers have noticed similar forms in human dysenteric stools,[2] some associated with

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[1] Jour. Inf. Dis., 1907, iv, 223.

[2] Chatterzee: Ind. Jour. Med. Res., 1917, iv, 393.

"Amœba coli." Similar forms have been seen also in an echinococcus cyst of the liver, in the sputum from a case of lung gangrene, in the exudate of a hydropneumothorax, and a few times in the urine. They are all probably harmless invaders though they may play a part in secondary infections.

## LEPTOMONAS, HERPETOMONAS, AND CRITHIDIA.

Certain flagellates found in the digestive tracts of mosquitoes, flies, and other insects are very similar to trypanosomes. Among them several species have been recognized, but they need to be more fully studied in order to determine their definite relationship to each other and to the genus trypanosoma. Leptomonas is described as having a single flagellum directed forward and arising near a blepharoplast situated in the anterior part of the cell. Herpetomonas is distinguished from leptomonas by a flagellum containing two filaments and by a delicate filament extending from the blepharoplast toward the posterior end. Crithidia has a rudimentary undulating membrane. The distinctions between these three genera and the genus Trypanosoma which have been recognized are: (1) the former contain no undulating membrane or only a rudimentary one, and (2) their centrosome or blepharoplast usually lies at the side of, or anterior to, the nucleus instead of posterior to it, as in Trypanosoma (Fig. 174).

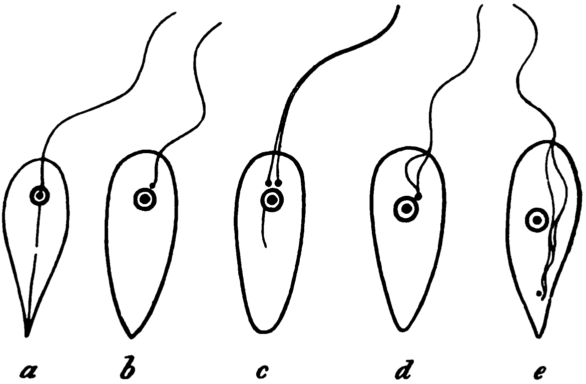

FIG. 174.—Schematic drawings of flagellates belonging to the trypanosomedæ, showing differential points: *a*, Cercomonas; *b*, Leptomonas and Leishmania; *c*, Herpetomonas; *d*, Crithidia; *e*, Trypanosoma.

These distinctions, Novy claims, may disappear in the cultural forms of the three genera, when trypanosomes may show a rudimentary undulating membrane and an anterior blepharoplast. His caution in regard to confusing these insect flagellates with developmental stages of vertebrate blood parasites should be remembered.

The Leishman-Donovan bodies found in kala-azar are closely related to the genus Leptomonas. They are considered a separate genus, Leishmania.

## LEISHMANIA (LEISHMAN-DONOVAN BODIES AND ALLIES).

Certain fevers of severe malaria-like types known in different sections of the tropics by different names (dum-dum fever, cachexial malaria, kala-azar) have been shown to have a causal relationship by the finding of similar protozoön-like bodies in the lesions. These bodies were first minutely described by Leishman, in 1903, as being present in certain cells in the spleen of cases occurring in India, called by him dum-dum fever. He considered them as possibly trypanosomes, but did not name them. Later in the same year Donovan described similar bodies in cases of what he called malarial cachexia. The bodies were first called the Leishman-Donovan bodies; then Laveran and Mesnil who examined Donovan's preparations and considered the organisms similar to those causing Texas fever in cattle, called them Piroplasma donovani. Ross, however, thought they constituted a distinct genus which he called Leishmania. This genus is now accepted, hence they are known as Leishmania donovani. Rogers and Patton place them with the genus Herpetomonas, but until we know more of the limits of variation of all these forms it seems best to make them a separate genus. They have since been found in different parts of India, in China, Tunis, Algiers, Arabia, Egypt, South Africa, Italy, Greece, and Portugal. Wright[1] in this country, has reported in a case of tropical ulcer, or Delhi boil, from an Armenian immigrant, bodies which, according to his excellent photographs (Fig. 175) and description, must be identical with, or very closely related to, Leishman's bodies. On account of the different pathological conditions in which they are found, however, they are classed as a different species, Leishmania tropica Wright. The form found in infantile splenomegaly is considered another

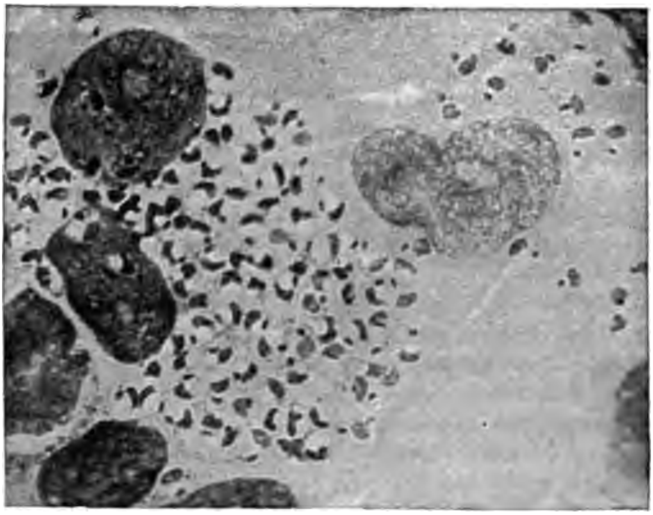

FIG. 175.—Leishmania tropica in a case of tropical ulcer. Smear preparation from the lesion stained with Wright's Romanowsky blood-staining fluid. The ring-like bodies with white central portions and containing a larger and a smaller dark mass are the microörganisms. The dark masses in the bodies are stained a magenta, while the peripheral portions of the bodies in typical instances are stained a pale robin's egg blue. The very large dark masses are nuclei of cells of the lesion. × 1500 approximately. (After Wright.)

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[1] Jour. Med. Res., 1903, x, 472.

species, with the name Leishmania infantum Nicolle.[1] The diseases caused by these organisms are classed under the name Leishmaniasis. Darling[2] described an organism resembling that of kala-azar found in a fatal disease of tropical America. Though the organism, he says, resembles donovani, he thinks it has enough points of difference to be placed in a different genus; therefore he gives it the name Histoplasma capsulatum, and calls the disease histoplasmosis. He says it differs from donovani in the form and arrangement of its chromatin nucleus and in not possessing a chromatin rod. It has a refractile achromatic capsule.

**Morphology.**—The bodies as seen in the cells of the host are circular to elliptical in shape, from $2\mu$ to $4\mu$ in diameter, and contain two nuclei, a large oval one at one part of the periphery and a small circular or rod-shaped one (blepharoplast) near or at the opposite part of the periphery. This smaller body stains more intensely than the larger one, while the cytoplasm of the parasite stains very dimly, sometimes showing only a faint peripheral rim. Near the rod-shaped nucleus may sometimes be seen a minute granule or rod which is the rudiment of the flagellum. Any nuclear and cytoplasmic staining methods will bring out these points in Zenker-fixed material. Smears stain well by Wright or the Nocht-Romanowsky methods. It was not until these organisms were cultivated outside of the body that their relationship to the flagellates was established (Fig. 176 and Plate IV, I, Fig. 1, B).

**Site in Body.**—The bodies have been found in large endothelioid cells in the spleen, liver, bone marrow, lymph nodes, kidney, lungs, testes, skin, muscles, intestinal ulcers and in the leukocytes in the peripheral blood. In this last situation they are only found in appreciable numbers in advanced cases.

The large cells containing the parasites are supposed by Christophers to be the endothelial cells from the finest capillaries. Donovan states that he found small forms in the polynuclear leukocytes of the peripheral circulation when the temperature was above 103°. Marshall found them chiefly in the mononuclears. Rogers was able to demonstrate them more easily in the peripheral circulation by centrifuging the blood and examining the leukocytic layer. Nicolle and Conte and others obtained them in the mononuclears found in the fluid of blisters produced experimentally on the skin.

**Cultivation.**—Rogers has grown abundant pure cultures of the bodies in a slightly acid citrated blood medium at 20° to 22° C. Nicolle and later Novy have shown that L. infantum is pathogenic for dogs and other animals and that cultures may be obtained with comparative ease from the infected animals (Fig. 176). Nicolle has also cultivated L. tropicum.

In these cultures the rounded organism elongates, the flagellum develops, and the Leishman-Donovan body becomes a flagellate, like the Leptomonas (Fig. 174).

[1] Ann. de l'Inst. Past., 1909, xxiii, 361 and 441.

[2] Jour. Exp. Med., 1909, xi, 515.

Recently Del Aguila[1] has reported the successful inoculation of the American Leishmania into the testicles of rodents.

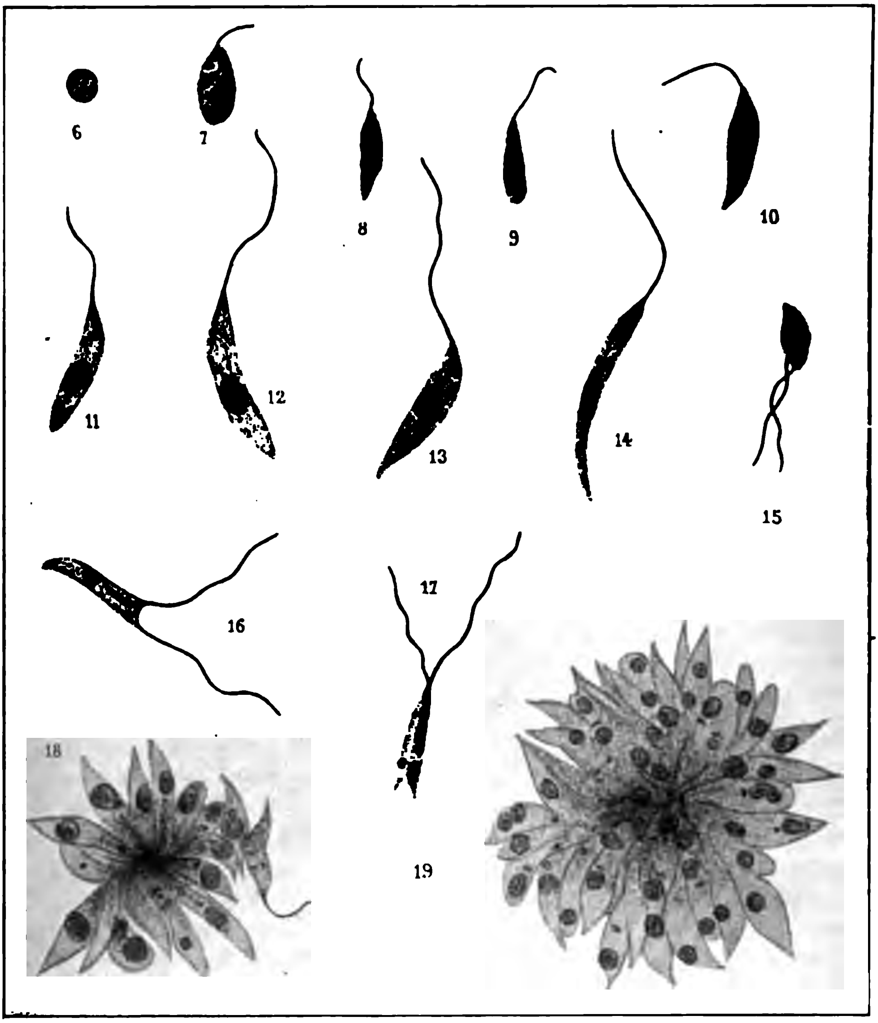

FIG. 176.—Cultural forms of *L. infantum*, showing the flagella type. (After Nicolle.)

**Insect Carriers.**—Rogers and Patton have shown that the bed-bug may transmit the disease, and Patton[2] has demonstrated the development of the organism up to the fully flagellated stage in the gut of this insect. The Sargents[3] have shown that L. infantum may be transmitted by the dog flea.

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[1] Anal. d. l. Facul. Méd., June, 1919, ii, 42.

[2] Scientific Memoirs by Officers of Medical and Sanitary Departments of Government of India, N. S. No. 31.

[3] Bull. Soc. Path. Exp., 1912, v, 595.

**Effect on Human Host.**—The pathological changes are those following the degenerations subsequent to the growth of the organisms in the large mononuclear cells. The *symptoms* in the cases of general infection are: (1) Very much enlarged spleen and less enlarged liver; (2) progressive anemia with peculiar, dark, earthy pallor of skin (kala-azar), progressive emaciation, and muscular atrophy; (3) long-continued, irregularly remittent, and intermittent fever (97° to 104°); (4) hemorrhages, such as epistaxis, bleeding from gums into subcutaneous tissue, producing purpuric eruption; (5) transitory edemas of various regions. There are often complications, such as congestion of lungs, dysentery, and cancrum oris. The blood shows practically no loss of red blood cells, but a diminution of hemoglobin; there is a decrease in the leukocytes, principally polynuclears, giving a relative increase of mononuclears.

Negative points which help in the diagnosis are: absence of malaria, no typhoid or Malta fever reaction, resistance to medication, quinine, as a rule, having no effect though in early cases and with large continued doses a few good results have been reported. Puncture of spleen or liver with the finding of Leishman-Donovan bodies makes the diagnosis certain. Sometimes the bodies may be found in the peripheral blood.

The duration of the disease is from a few months to several years. The percentage of deaths in systemic infections is great; in some forms of the disease at the height of an epidemic it may reach 98 per cent. Natives seem to be more susceptible than strangers from non-tropical countries. The infection in children known as splenomegaly is similar to that in the adult.

The local disease known as Delhi or Aleppo boil or tropical ulcer is a comparatively non-dangerous circumscribed chronic ulcer in which the endothelial cells contain the organisms in large numbers (Fig. 174). Recovery is followed by marked immunity.

*Complement Fixation.*—Makkas and Pappassoterion[1] state that a specific antigen gives positive results with both kala-azar and syphilis, but a syphilitic or a non-specific antigen gives positive results only with syphilis, therefore both should be used.

**Prophylaxis.**—Segregation and perfect cleanliness, especially in regard to bed-bugs and fleas, are recommended as the best means of eradicating the disease.

*Curative Treatment.*—Continued favorable reports are coming of the use of tartar emetic introduced by Machado and Viamia in Brazil in both superficial[2] and internal[3] Leishmaniasis.

**Uta.**—A South American disease known under several terms, Espundia and others, has been shown by Strong and his co-workers[4] (1913) to be due to a species of Leishmania. The flagellate stage of the organism was obtained and animals were successfully inoculated from a human case.

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[1] Arch. d. Med., 1911.

[2] Da Malta Amaz. Med., 1918, i, 13.

[3] Pastore, Pediatria, 1919, xxvii, 96.

[4] Jour. Am. Med. Assn., 1913, lxi, 1713.

# CHAPTER XXXVIII.

## TRYPANOSOMA.

**Pathogenic Forms.**—Very many species of trypanosoma have been described, and the number reported as distinctly pathogenic is increasing. Two of the latter are known to be pathogenic for man; a closely related form that was described by Chagas in 1909 is made a new genus. The table on page 549 gives a list of the better-known forms pathogenic for mammals with their chief differential characteristics. They are divided by Laveran into three groups according to the different characters of the flagellum. Their general characteristics and broader classifications are given in Part I. (See also Plate IV, 1, Fig. 2.)

**Historical Note.**—The first species of trypanosome studied with any degree of fulness is the comparatively non-virulent T. lewisi. It was probably first seen in the blood of the rat in 1845, but was not well described until 1879, when Lewis studied it more fully. Since then it has been studied by many observers. It is found in the blood of from 2 to 3 per cent. of wild rats throughout the world.

The first of the more pathogenic trypanosomes was discovered by Evans in the blood of East Indian horses suffering from surra, but it was not well studied until 1893, when Lingard's important work on *surra* led, in a way, to all the subsequent work on diseases caused by trypanosomes. The next year a trypanosome was discovered by Bruce[1] in the blood of horses and cattle suffering from nagana in Zululand and other parts of Africa. Bruce further demonstrated the important fact that the disease was transmitted by the bites of flies, the tsetse flies (*Glossina*). Announcements of other pathogenic trypanosomes in different parts of the tropics quickly followed. In 1896 Rouget found that *dourine*, a disease of equines in Algiers and South Africa, was caused by a trypanosome (T. equiperdum). Then the South African disease of horses, called *mal de Caderas*, was shown by Voges to be due to a similar flagellate, while in 1902, Theiler found a variety of trypanosome in the blood of cattle in the Transvaal suffering from the disease called *galziekte*, or gall sickness. The number of trypanosomes found in the tropics is constantly increasing—both pathogenic and non-pathogenic forms.

Man was thought to be comparatively immune to trypanosomes until the important discovery was made that trypanosomes are the specific cause of a definite disease known as *sleeping sickness*, which occurs chiefly in the African negro. In 1898 Nepvieu reported having found trypanosomes in the blood of 6 out of more than 200 cases of human beings examined for malarial organisms and in a seventh case which was apparently in good health. The eighth case is reported by Dutton in 1901. The tenth and eleventh cases were published by Manson in 1902, Bröden published 2 more cases, and Baker 3. In 1904 Castellani stated that the cause of sleeping sickness of the negro is a trypanosome. He found trypanosomes in the centrifugalized cerebrospinal fluid of 20 out of 34 cases of this disease. His work has been fully corroborated. The trypanosomes found in these cases resemble each other; they are therefore included under the same name, Trypanosoma gambiense Dutton. A similar form found by Stephens and Fantham[2] in 1911 in cases of sleeping sickness in

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[1] British Med. Jour., 1915, Nos. 2843-2846.

[2] Proc. Roy. Soc., 1910, lxxxviii, 28.

DIFFERENTIAL CHARACTERISTICS OF MORE IMPORTANT TRYPANOSOMES PATHOGENIC FOR MAMMALS (MODIFIED FROM YORKE AND BLACKLOCK).

| Groups. | Sub-groups. | Species. | Date discovered. | Where first found. | Size in microns. |  |  |  | Other characteristics. | Pathogenesis. |  |  | Invertebrate host. |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  | Length. |  | Breadth. |  |  | Vertebrate host. | Name of disease. | Laboratory animals. | Name. | Place of development. |
|  |  |  |  |  | Max. | Min. | Max. | Min. |  |  |  |  |  |  |
| I. Always free flagellum. | 1 | T. lewisi Kent | 1881 | Throughout world | 38 | 10 | 3.0 | 1.5 | Very motile, nucleus at anterior and middle third, blepharoplast rod-shape. | Rats | Trypanosomiasis | Rat slight | Rat fleas (Ctenophthalmus) and lice (Hæmatopinus) | Intestines. |
|  |  | T. evansi Steele | 1885 | India | 34 | 18 | 2.0 | 1.5 | ............ | Equidæ, cattle, camels | Surra | Acute | Stomoxyx (fly) | Unknown. |
|  | 2 | T. brucei Plimmer and Bradford | 1899 | Zululand | 34 | 18 | 2.5 | 1.5 | Post. end usually bluntly rounded | Equidæ, cattle | Nagana | Acute | Glossina | Gut and salivary glands. |
|  |  | T. hippicum Darling | 1910 | Central America | 28 | 18 | 3.0 | 1.5 | ............ | Equidæ | Trypanosomiasis | Acute | ........... | Unknown. |
|  | 3 | T. equiperdum Doflein | 1901 | North Africa | 35 | 16 | 3.0 | 1.5 | ............ | Equidæ | Dourine | Acute | Transmitted by coitus. |  |
|  | 4 | T. vivax Ziemann | 1905 | Cameroon | 31 | 16 | 3.0 | 2.0 | Very motile, club shaped | Equidæ, cattle | Trypanosomiasis |  | Glossina | Proboscis. |
|  | 5 | T. theileri Laveran | 1902 | German East Africa | 70 | 25 | 5.0 | 2.0 | Very large | Equidæ, cattle | Sleeping sickness |  |  |  |
|  | 6 | T. (Schizotrypanum) cruzi Chagas | 1909 | Brazil | Average |  | 20.0 |  | Dividing forms not seen in blood, blepharoplast large | Man, monkey | Trypanosomiasis | Slight | Conorhinus | Gut and salivary glands. |
| II. Never free. | 7 | T. congolense Borden | 1904 | Congo | 19 | 8 | Average | 1.5 | ............ | Equidæ, ruminants | Trypanosomiasis | Acute | Glossina | Proboscis. |
|  | 8 | T. simiæ Bruce | 1912 | Nyasaland | 23 | 12 | .. | .. | ............ | Monkeys | Trypanosomiasis | Acute for monkeys and pigs | Glossina | Proboscis |
| III. Free or not. | 9 | T. gambiense Dutton | 1902 | Gambia | 39 | 13 | .. | .. | ............ | Man | Sleeping sickness | Acute | Glossina | Gut and salivary glands. |
|  | 10 | T. equi Blacklock and Yorke | 1913 | North Africa | 36 | 14 | .. | .. | Posterior nuclear forms | Equidæ | Sleeping sickness | Acute | Transmitted | by coitus. |

Rhodesia has been given another specific name, T. rhodesiense, chiefly because of the characteristic action in experimental animals.

Chagas, in 1909, states that a trypanosome which he had discovered in a small monkey (Callethrix hapalepenecellata) is the cause of human infection (Chagas' disease) in Rio de Janeiro. Because of its ability to grow in the tissues of infected animals it is classified by some as a new genus—schizotrypanum. It is carried by a hemiptera, genus Conorrhinus. The flagellate is small with a large blepharoplast (kinetonucleus). It grows on blood agar readily and infects laboratory animals easily. Chagas reports developmental forms in the monkey's lung and in the gut of the fly. Chagas has also reported that this trypanosome causes thyroiditis in children. Tejera[1] reports similar infections in Venezuela, transmitted by a different insect (Rhodimus prolixus.)

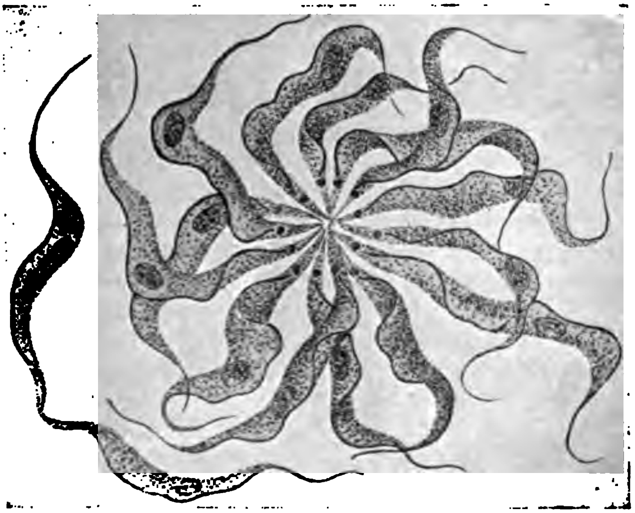

Fig. 177.—Agglutination of Trypanosoma lewisi. (Laveran and Mesnil.)

**Comparative Characteristics of the Different Species.**—The form changes of the same species in the same host are so varied that few have been found absolutely characteristic of a single species, and, as physiological properties alone are not considered final in species classification, we cannot be sure that all of the organisms in this group described as separate species are so until more of the complete life histories are known. Until then each new form found with distinct physiological properties, though apparently morphologically similar to others, may expediently be considered a new species.

As an example of the difficulty of deciding whether or not a trypanosome found is a new species, the history of T. rhodesiense may be taken. All of the points brought forward by Stephens and Fantham, the discoverers, as evidence of a new species have been combated by others; and its relation to T. gambiense on one side and T. brucei on the other is an open question. At present the points are in favor of its being the same as T. brucei.

**Morphology.**—**Size.**—The variations recorded in the *dimensions* of the species we are considering may be seen by glancing at the foregoing table.

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[1] Gac. Méd. d. Caracas, 1919, x, 103.

The trypanosomes pathogenic for man (*T. gambiense* and *T. rhodesiense*) have the smallest average size of the group. With the exception of *T. theileri* and *T. transvaaliense* (not given in table), which are much larger than any other of these forms, the variations in size of the different species are not so marked as they are in the same species under different conditions.

**Shape.**—In shape, though all follow the type, each species varies greatly according to conditions of growth and multiplication. At times they may be slender and worm-like, at others they may be so short and thick as to be almost round. *T. lewisi* has the posterior (aflagellar) end often thinner and more pointed than the other species. *T. evansi* is generally a little longer and thinner than *T. lewisi*, while *T. brucei* has a more rounded aflagellar end than either, and is generally broader, more club-shaped (Plate IV, Figs. 1, 2).

**The Cytoplasm.**—The cytoplasm differs slightly in the different forms. *T. lewisi* is relatively free from chromatoid granules, while *T. brucei* has usually many. Myoneme fibrils have been demonstrated in some species and probably all contain them. An oval vacuole has been seen in some species.

**The Nuclear Apparatus.**—The nuclear apparatus is essentially similar in all forms. The two nuclei (tropho- and kinetonucleus) vary somewhat in position and size in the different species and at different stages in the same species. In *T. theileri* and in young forms of *T. lewisi*, both nuclei lie close together near the center of the organism. In *T. lewisi* the trophonucleus is situated more anteriorly than in the other species. In *T rhodesiense* the nucleus is often posterior to the center, especially in experimental animals.

Many variations from the type forms are seen. Some are no doubt degeneration and involution forms. *Three* forms, however, which are more or less constantly seen in all the species have been interpreted as definite phases in the life cycle. These forms were first described by Schaudinn in T. noctuæ, and were interpreted by him and since then by others as male, female, and indifferent form. The male cells are smaller, more hyaline, and more free from granules than the female. The nucleus of each sexual cell rids itself of male and female chromatin, respectively. The indifferent cell, on the contrary, has a complete nucleus. Opinions differ as to Schaudinn's interpretation being the correct one. More research is needed before we can arrive at a definite conclusion.

**Motility.**—The first thing noticed on examining a fresh hanging drop of blood at a magnification of 100 to 300 diameters is active movements of the red blood corpuscles in certain areas, and, on carefully focussing over one of these areas, the rapidly wriggling worm-like organism may be seen. As the movements become slower, the flagellum may be seen swaying from side to side and the wave-like movements of the undulating membrane are quite discernible. Movement is twofold: (1) progression with an auger-like motion effected by the undulating membrane assisted by the flagellum; (2) contractions of the body assisted no doubt by myoneme-like structures. Relatively, *T. lewisi* is most active and *T. brucei* least. Motility soon ceases outside of the body, continuing longer if the organism has been kept in the ice-box than at higher temperatures. Aflagellar forms, sometimes ameboid, have been frequently described in the blood of mammals.

**Reproduction.**—The usual method of multiplication is binary longitudinal fission. In several species a rosette-like segmentation has also been observed. Longitudinal fission begins usually with division of the kinetonucleus, then of the trophonucleus and cytoplasm; but this order of division seems to be quite variable. The flagellum often appears to be dividing first, and probably division always starts with the centrosome-like basal granule of the flagellum. In many cases a new flagellum seems to be formed instead of division of the old one. The details of division have not been frequently studied, but it is probable that both nuclei divide by a primitive mitosis. During division the kinetonucleus generally moves near the trophonucleus. Generally the fission is equal, but occasionally the daughter trypanosomes may be quite unequal in size. This is notably the case in division of *T. lewisi* where the cytoplasm may divide so unequally that the process may be compared to budding. The resulting small parasites have at first no undulating membrane, hence they resemble somewhat Leptomonas. These young forms may divide several times in succession, producing smaller and smaller fusiform parasites. As a result some forms are so small that they can only be seen when agglomerated or in motion (Schaudinn). The question as to whether trypanosomes undergo phases of development in their invertebrate hosts has been widely studied. Prowazek has described sexual forms of *T. lewisi* in the body of the louse. The subject, however, requires further research for convincing evidence.

**Insect Carriers.**—The trypanosomiasis of vertebrates is transmitted by blood-sucking insects (dourine is possibly an exception). Bruce (1894) first showed that *T. brucei* was conveyed by the fly *Glossina morsitans*. Since then other varieties of flies also have been shown to spread the disease (table, p. 549). Among them *Glossina palpalis* (Fig. 178) is supposed to be the chief agent in transmitting human trypanosomiasis. These flies bite by day and in full moonlight. The infective period of the insects after they have bitten a sick animal is variable. Bruce found living trypanosomes in the proboscides of the flies up to forty-eight hours. Up to one hundred and eighteen hours he found them in the flies' stomachs, after one hundred and forty hours he found the stomachs empty, and what appeared to be dead parasites in the excreta. Klein and others have since found that after a certain number of days (usually from twelve to twenty-five) a small percentage of the biting flies which were fed sufficiently on an infected animal become infective again. This is due to the fact that the trypanosomes have developed in the gut and have passed to the salivary glands of the fly. They remain in the glands probably during the life of the fly, and continue to be infective (Bruce, Hammerton, Bateman, Mackie,[1] and others).

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[1] Proc. Roy. Soc., 1911, lxxxi, 405.

Dourine in horses is usually communicated during the act of coitus. Surra may be transmitted to dogs by eating infected animals. The rat trypanosome is conveyed by contaminative infection through the dejecta of the fleas or lice when biting a new host. (See also under *historical note.*)

**Cultivation.**—Novy and MacNeal[1] were the first (1903) to cultivate trypanosomes in the test-tube. They have grown *T. lewisi* through many culture generations extending over several years. At the last tests made the parasites were as virulent as at the beginning. The culture medium used in their work was the condensation fluid from slant tubes of ordinary nutrient agar containing variable amounts of fresh defibrinated or laked rabbit or rat blood. The best results were obtained with a mixture of equal parts of blood and agar. At room temperature the growth is slower but surer than in the thermostat. A culture at room temperature retains its vitality for months; thus in one case the trypanosomes were alive after three hundred and six days. Novy and MacNeal also cultivated *in vitro T. brucei, T. evansi,* and various bird trypanosomes. The latter they found especially easy to cultivate, while the former are much more exacting in their requirements than is the *T. lewisi.* They require two parts of blood to one of agar, and growth is best at 28° C. The primary cultures are not transferred until the end of three weeks. These primary cultures are not always virulent for animals, but subcultures regain their virulence.

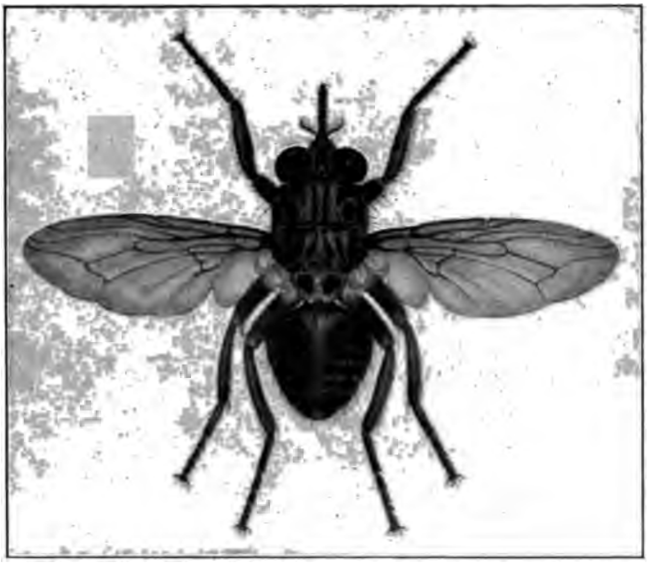

FIG. 178.—Glossina palpalis, carrier of the human trypanosomiasis caused by T. gambiense. × 4. (Kolle and Wassermann.)

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[1] Jour. Inf. Dis., 1904, i; 1905, ii, 256.

Mice and rats die in six to fourteen days after inoculation. Guinea-pigs have a relapse in two to ten weeks.

The great majority of trypanosomes experimented with since the initial investigations, have been found by various workers to be cultivatable, with more or less ease. *Trypanosoma gambiense,* however, seems to be more difficult to cultivate (Thompson and Sinton[1]).

**Effect on Vertebrate Host (Pathogenesis).—Lower Animals.**—Many of the lower vertebrates have become, through mutual toleration, natural hosts of the trypanosome. It is probable that each pathogenic trypanosome has an indigenous wild animal as natural host and that in this way the supply to strange mammals coming into the vicinity is kept up. These strange animals, being unaccustomed to the native trypanosomes, often succumb to the infection.

**Symptoms.**—In general the descriptions given of the symptomatology of trypanosomiasis in various animals show a great similarity, though there is much variation in individual cases. The average clinical picture, according to Musgrave and Clegg,[2] is as follows: After an incubation period which varies in the same class of animals and in those of different species, as well as with the conditions of infection, and during which the animal remains perfectly well, the first symptom to be noticed is a rise of temperature. For some days a remittent or intermittent fever may be the only evidence of illness. Later on the animal becomes somewhat stupid; watery, catarrhal discharges from the nose and eyes appear; the hair becomes roughened and falls out in places and the peripheral lymph nodes are enlarged. Finally, the catarrhal discharges become more profuse and the secretions more tenacious and even purulent; marked emaciation develops; edema of the genitals and dependent parts appears; a staggering gait, particularly of the hind parts, comes on, in some forms passing on to paralysis. This is followed by death. There may be various ecchymoses and skin eruptions. Parasites are found in the blood more or less regularly after the appearance of the fever. They are often more numerous in the enlarged lymph nodes and in the bloody edematous areas than in the general circulation.

The *autopsy* shows general anemia, an enlarged spleen with hypertrophied follicles, more or less gelatinous material in the adipose tissue, the liver slightly enlarged, a small amount of serous exudate in serous cavities, edematous condition, and small hemorrhages in various tissues. There is a relative increase of the mononuclears in the blood.

The *duration* varies from a few days to many months. The *prognosis* seems to be influenced to a certain extent by the species of host. It is probably always fatal in horses. Some cattle recover. The chief cause of death is possibly a toxic substance, though no definite toxin has been isolated. Mechanical disturbances (emboli, etc.) also probably play a part in producing death.

**Man.**—*Sleeping sickness,* or *human trypanosomiasis,* is an endemic disease in certain regions of equatorial Africa. Neither age nor sex are predisposing factors, but occupation and social position seem to have a marked influence, the great majority of cases occurring among very poor field workers. As these workers are all negroes, the question of the relative influence of race cannot be determined. The white race, however, is not immune, as has been frequently shown.

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[1] Ann. Trop. Med., 1912, vi, 351.

[2] Trypanosoma and Trypanosomiasis, etc., Manila, Bureau of Public Printing, 1903.

T. gambiense, the trypanosome first shown to be pathogenic for human beings, is irregularly pathogenic for some monkeys (Macacus rhesus and others), for dogs, cats and rats. It is less pathogenic for mice, guinea-pigs, rabbits, horses, baboons, cattle, and swine. In fact it has a very wide range of pathogenicity.

Flies (Gl. palpalis) have been found to be infected with this trypanosome in areas which have had no human population for several years, a longer time than the life of a fly. And since trypanosomes are not known to be hereditarily transmitted, it is concluded that certain mammals are harboring the trypanosomes, thus acting as reservoirs.

**Symptoms.**—The course of the disease is very insidious, as the trypanosomes may exist in the blood for a long time before entering and growing in the cerebrospinal fluid and causing the characteristic symptoms. Therefore the symptoms may be divided into two stages. In the first stage there is only an irregular fever with enlargement of the peripheral lymph nodes. In the second stage the fever becomes hectic, the pulse is constantly increased; there are neuralgic pains, partial edemas and erythemas, trembling of the muscles, gradually increasing weakness, emaciation, and lethargy. The somnolence increases until a comatose condition is developed and death occurs. In the second stage trypanosomes are always found in the cerebrospinal fluid. Throughout the disease they are usually found in small numbers in the blood.

**Duration.**—The first stage may last for several years; the second, from four to eight months. The percentage of deaths in cases reaching the second stage is 100. Whether some in the first stage recover is not yet certain.

**Pathological Changes.**—Congestion of the meninges; increased quantity of cerebrospinal fluid; hypertrophy of spleen, liver, and lymphatic ganglia; diminished hemoglobin and number of red cells; number of leukocytes about normal, but a relative increase of eosinophiles, mast cells, and lymphocytes. Enlargement of the superficial lymph nodes has been noted as an early symptom and has thus been made use of in diagnosis. Dutton and Todd found that 91 per cent. of natives in the Congo Free State, who had posterior cervical glands enlarged, showed trypanosomes in the punctured gland juice.

**Diagnosis of Trypanosomiasis in General.**—This should be made as early as possible in order to prevent the spread of the disease. An early positive diagnosis can only be made by the determination of the peripheral infection. This is done in two ways: first, by microscopic examination of freshly drawn blood, cerebrospinal fluid, or tissue from enlarged peripheral lymph nodes; second, by animal inoculation of the blood or other tissue. In the microscopic examination it may be necessary to examine the blood of the suspected animal for several days in succession. The parasites are rarely absent in the early stages in domestic animals for more than a few days at a time, while in man the time may be much longer.

**Methods of Examination.**—BLOOD.—If the direct examination of a drop of the blood is negative, 10 c.c. should be withdrawn from the vein, and after adding a tenth of its volume of 10 per cent. citrate of sodium it should be centrifuged for ten minutes, and the sediment examined in hanging drop and in smear. The great majority of the parasites will be found collected with the white cells in the thin white layer between red blood cells and serum, which may easily be removed with a fine pipette. The parasites are readily detected with the low power of the microscope (× 100) by areas of irregularly moving cells. If only a small amount of blood can be obtained, the tiny tubes recommended by Wright in his opsonin work may be used.

CEREBROSPINAL FLUID.—Ten c.c. of the fluid withdrawn by lumbar puncture should be centrifuged for fifteen minutes, and the deposit should be examined under 150 to 200 diameter magnification.

*Cultures* sometimes give positive results, especially from infections in lower animals and in infections with *T. rhodesiense*.

THE INOCULATION TEST.—If the trypanosomes cannot be found by the above methods, animal inoculation should always be made. Monkeys, if possible, should be used, or if monkeys cannot be obtained, dogs or rats may be used. A few drops to 1 c.c. of the blood or other tissue from the suspected animal should be inoculated intraperitoneally or subcutaneously.

Smears may be stained by any modification of the Romanowsky method. Giemsa's method gives good results (Plate IV, Figs. 1, 2).

**Prophylaxis.**—The disease is readily controlled by preventive measures. There should be strict quarantine regulations governing the importation of animals. When the disease has once appeared, the following general measures should be taken: (1) Suspected animals should be isolated. (2) All infected animals should be destroyed. (3) As far as possible, all biting insects should be destroyed, and the land in the neighborhood of human habitation should be suitably cultivated. (4) The bodies of infected animals should be protected from biting insects for at least twenty-four hours after death. (5) Susceptible animals should, if possible, be made immune. (6) All means possible should be used to exterminate the reservoirs. In sparsely settled districts the natives may be removed from the fly area.

**Treatment.**—The whole question of treatment is still in the experimental stage. The chronic course of the disease with relapses often after long intervals makes it impossible, especially in cases of human trypanosomiasis, to come quickly to a conclusion in regard to the efficiency of any drug. Many drugs have been found to possess trypanocidal properties to a certain extent.

Atoxyl (p-amino-phenyl-arsenic acid), introduced by Thomas (1905) and used first by Thomas and Breinl in treatment of experimental trypanosomiasis, proved to have a beneficial effect in the different forms of this disease.

The good reports received from the use of other arsenic compounds introduced by Ehrlich,[1] namely salvarsan and neosalvarsan, have continued.

**Serum Therapy.**—Various normal sera from different animals have been tried with practically no success. A few have prolonged life. Thus Laveran and Mesnil[2] state that human serum injected in sufficient quantities shows manifest action on the disease, and that sometimes cure results in mice and rats. Further, by alternating human serum with arsenic they obtained still better results. Kanthack, Durham, and Blandford showed that animals recovering from trypanosoma infection were immune to further infection. Rabinowitsch and Kempner have made a very careful study of immune serum produced by *T.*

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[1] München. Med. Wchnschr., 1909, v, 217.

[2] Trypanosomes and Trypanosomiases, Trans. by Nabarro, London, 1907.

*lewisi*. They have shown that an animal may be hyperimmunized and that then its serum, in comparatively large doses, inoculated into mice at the same time as the trypanosomes, or twenty-four hours before or after, allows no development of the organisms. Laveran and Mesnil state that the serum causes the rapid destruction of the organisms by the leukocytes, though MacNeal,[1] on the other hand, states that the trypanosomes are destroyed by a cytolytic action of the serum. This immune serum also has a similar action on the trypanosoma of dourine. The serum of animals hyperimmunized against other varieties of trypanosoma is not as active as that obtained by the inoculation of *T. lewisi*. Koch suggested that immunity might be produced by the inoculation of attenuated parasites, and Novy and MacNeal have succeeded in attenuating cultures of *T. brucei*, and have obtained some success in protecting experimental animals against virulent culture.

**Complement Fixation.**—The earlier tests did not promise practical results. Several investigators, however, have given more favorable reports. In this country Mohler claims that the test is of great worth in diagnosing dourine, many cases of which have occurred in some of our western States (notably Iowa and Montana). Mohler, Eichhorn, Buck and Traum[2] state that they use a fresh antigen prepared from the spleens of rats dead after infection with surra (used because it is easier to transmit than is dourine). The antigen is simply a filtered emulsion in salt solution of the infected spleen. The emulsion from each spleen is made up to 40 c.c., by addition of salt solution. A fresh antigen must be prepared and titered (see Part I) each day. The results have been controlled by autopsies of animals giving a positive reaction.

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[1] Jour. Inf. Dis., 1904, i, 537.

[2] Jour. Agric. Res., 1913, i, 99.

# CHAPTER XXXIX.

# SPIROCHETA AND ALLIES.

THE genus *Spirocheta* was introduced by Ehrenberg in 1838, who differentiated it from spirillum by its flexibility. Schaudinn, in 1905, thought he saw an undulating membrane in *Spirocheta refringens*, so he added this characteristic to the genus and considers that thus its relationship to the flagellated protozoa, genus *Trypanosoma*, is indicated.

Since the appearance of the work of Schaudinn and Hoffmann[1] (1905), showing the etiological relationship of a spirochete to syphilis, the *Spirochetæ* have been brought into great prominence.

Numerous spirochetes and spiral organisms have been described, some associated with *Spirocheta pallida* (*Treponema pallidum*) in syphilis, some in other lesions or in the normal secretions of both man and the lower animals; and still the question as to their classification is unsettled. The majority of observers, however, are willing to admit that—the structure of many of the varieties classed with this group is more complicated than that of bacteria and that hence the group may be an intermediate one between protozoa and bacteria.

The chief reasons given for considering spirochetes protozoa are: (1) the flexibility and the indications in many of longitudinal division and of undulating membrane: (2) the demonstration of forms intermediate between the trypanosomes and the spirochetes; (3) the spirochetal forms of certain trypanosomes (T. noctuæ); (4) stages of development in the louse and tick; (5) dissolution by certain chemicals such as saponin or sodium taurocholate in contrast to the resistance shown by most bacteria.

In favor of the bacterial nature of spirochetes are: (1) the rigidity of some forms, the lack of undulating membrane in most and of definite nuclear apparatus in all, and the evidence of transverse division in all and of flagella arising from the periplast in some; (2) the cultivation of certain forms (*e. g.*, Sp. refringens, by Levaditi; Sp. obermeieri by Novy; several spirochetes by Noguchi and others) for many generations without development of trypanosome forms.

The classification then of the group of organisms commonly called spirochetes is still undecided. Noguchi[2] in his recent Harvey lecture goes into details of the various groupings proposed. The first classification quoted is given here:

### CLASSIFICATION AFTER GONDER.

Spironemaceæ (Gross, 1910).

| | |
|---|---|
| Spirocheta . . . (Ehrenberg, 1838) | Type: Spirocheta plicatilis; all free living. |
| Cristispira . . . (Gross, 1910) | Type: Cristispira balbianii, and other varieties found in mussels. |
| Spironema . . . (Vieillemin, 1905) | Type: Spironema recurrentis, and other parasitic varieties living in blood. |
| Treponema . . . (Schaudinn, 1905) | Type: Treponema pallidum, and other varieties with closely set spirals. |

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[1] Arbeit a. d. Kaiserl. Gesundh., 1905, xxii.

[2] Jour. Exp. Med., 1909, xi, 84 and 392; Ibid., 1911, xiv, 99; 1912, xvi, 194, 199, 211, 261, 620. Herter Lecture, 1915–1916, Philadelphia.

It may be well to note briefly the chief characteristics of the more familiar non-pathogenic species in order better to understand the relationships between them and the *Treponema pallidum* and other pathogenic forms.

**Material and Methods for Study.**—A large spiral organism (cristispira balbianii) closely related to the spirochetes is found in the stomach of oysters fresh from salt water. Smaller spirochetes are frequently found in human mouths. When fresh syphilitic or relapsing fever material can be obtained this should be examined. The Treponema pallidum (the spiral organism of syphilis), because of its low refractive index, is seen when alive with difficulty by the ordinary microscope, but with the dark-stage illumination, especially if a drop of distilled water is added to the serum containing the organisms, it is seen distinctly and its motion and structure may be more easily studied. The fluid containing the organisms should be dropped on an ordinary glass slide, covered with a thin cover-glass, and well sealed with vaselin, as most spirochetes are anaërobic. Material may be obtained from syphilitic lesions as follows: The lesion is first thoroughly washed and dried with distilled water and sterile gauze. Part of the base and margin is then scraped with a curette until the superficial tissue is removed and blood appears. The blood is wiped away with sterile gauze until clear serum begins to ooze. A drop of this serum is used for examination.

Smears should be made as thin as possible and may be stained (1) by the method recommended by Giemsa (Tr. pallidum stains reddish). A modification of Giemsa, used by Schereschewsky[1] has been highly recommended by various workers; (2) by Goldhorn's methods.

**Cultures.**—Pure cultures have been obtained of the Treponema dentium in the following manner: Poured serum-agar plates are made of various dilutions of material from the mouth containing these spirochetes. After being kept in the thermostat at 37° C. under anaërobic conditions for nine to twelve days the spirochetal colonies are fished and planted in agar tubes as stick cultures.

Pure cultures of Spironema recurrentis (Spirocheta obermeieri) by Novy and of Spironema refringens by Levaditi have been obtained by growing in collodion sacs. (For other culture experiments see below.)

**Cristispira Balbianii (Certes).**—This immense form next largest known to the Spirocheta plicatilis Ehrenberg, may be found in the oysters' crystalline style. It is important for study because it is apparently a transitional form. In fact it is considered a trypanosome by Perrin[2] and others. Mühlens[3] gives its characteristics as follows: Length 26$\mu$ to 120$\mu$, in width $\frac{1}{2}\mu$ to 3$\mu$. The body is flattened and possesses a ridge, or crysta, which is visible during life on some individuals. It has 4 to 8 flat, wide spiral coils. Its movements are lively, similar to those of trypanosomes, but more corkscrew-like. During motion its form is apparently easily changed. The rim of the membrane-like ridge does not end in a free flagellum, but one end of it seems to be attached to a triangular mass of chromatin (basal granule, blepharoplast?) which is a part of the central chromatin material. The nuclear material is arranged in a more or less spiral band along the entire center of the organism.

Before division this nuclear band, after passing through chromosome-like changes, breaks up into pairs, and division takes place longitudinally between them. Division is often incomplete for a time, the two ends remaining attached.

**Spironema Balanitidis.**—This is a spirochete found by Simon in Balanitis circinata and regarded by some as the specific cause of this disease. Hoffmann and Prowazek[4] describe it as a rather strongly refractive, actively motile, band-shaped organism, shorter and thicker than Treponema pallidum, with 6 to 10 coils staining bluish red with Giemsa's method and at either end a periplastic cilium. No definite undulating membrane has been demonstrated.

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[1] Centralbl. f. Bakt., 1908, xlv, 91.

[2] Arch. f. Protist., 1906, vii, 131.

[3] Ztschr. f. Hyg., 1907, vii, 405; Klin. Jahrb., 1910, 339; Kolle and Wassermann, 2d ed.

[4] Ztschr. f. Hyg., 1911, lxviii, 27.

Mühlens thinks this may be identical with Spironema refringens. Levaditi has reported cultivating it (see below under Treponema Pallidum).

**The Mouth Spirochetes.**—Several species of non-pathogenic forms are commonly found in normal mouths.

1. *Spironema Buccalis* (Cohn).—Length, $10\mu$ to $20\mu$; thickness, $\frac{1}{3}\mu$ to $\frac{2}{3}\mu$. It has 3 to 10 irregular flat coils. No true cilia have been demonstrated. The claim of Schaudinn, Hoffmann and Prowazek that it has an undulating membrane has not been corroborated. It stains violet with Giemsa.

2. *Treponema Dentium* (Koch[1]).—This is much smaller than the previous form. It is as thin as the pallidum and is somewhat similar to it in refraction, staining qualities, and in the fixity of its coils during motion. It is somewhat smaller than the pallidum and stains a little more easily with Löffler's flagella stain, and flagella have been demonstrated. Neither definite undulating membrane nor nuclear material has been seen. It is $4\mu$ to $12\mu$ long, and has 4 to 20 regular spirals of about the same appearance as those of the pallidum. Pure cultures have been made from this spirochete as described above. Several varieties have been described.

3. *Treponema macrodentium*, a form in appearance between these two has been found in the mouth. This also is somewhat similar to the pallidum, but it is larger and has less regular spirals; moreover, it stains more intensely with the blue of Giemsa, only in poorly prepared specimens does it appear red.

**Spironema Refringens.**—Spironema refringens is also found in the mouth, but it is especially interesting because of the fact that it is so often found associated with the Treponema pallidum in the various lesions of syphilis. It is not in such large numbers as the pallidum and probably bears the relation of a restricted secondary invader. It is generally longer than the pallidum ($10\mu$ to $30\mu$) and much thicker ($\frac{1}{2}\mu$ to $\frac{3}{4}\mu$). In life it is much more refractive. It has 3 to 15 irregular wide, flat spirals which change their shape during motion. Its movements are much more lively than those of pallidum. With Giemsa it stains quickly and easily, a blue to a blue-violet tone, according to the length of staining. Schaudinn states that it possesses an undulating membrane. Levaditi claims to have demonstrated terminal cilia for this organism and to have cultivated it in collodion sacs in the rabbit's peritoneum.

**Spironema Vincenti.**—Accompanying the fusiform bacilli in Vincent's angina (see p. 501) are many spirochetes similar to the "middle form" found in the mouth. Whether they are identical with these spirochetes or whether they are a special variety (or, as some think, a second form of the fusiform bacillus) still remains to be determined. Their relationship to the disease is also uncertain.

**Spirochetes in Tumors.**—Löwenthal, Borrel, and others found spirochetes in small numbers in certain mouse tumors. Ewing and Beebe[2] found a few in some dog tumors and others have reported their occasional presence in both ulcerating and non-ulcerating human tumors, but apparently never in sufficient numbers to account for the tissue reaction. Gaylord, however, found that in repeated transplants of a mouse tumor, as the inoculated material became more virulent the number of spirochetes greatly increased. Calkins[3] studied the morphology of Gaylord's spirochete and decided it to be a distinct species. He has also found this species in primary as well as in transplanted tumors. It is much shorter and thicker than the pallidum, and has blunt ends. It closely resembles the spirochetes found comparatively frequently by Tyzzer and others in apparently normal mice, though the possibility of infection in these cases was not ruled out.

**Miscellaneous Spirochetæ.**—Besides the spirochetes found in syphilis, in frambesia, and the spiral organisms causing African and European relapsing fevers, all of which will be described below, spirochetes have been found (1) in the normal intestinal tract of mosquitoes and human beings as well as in the diarrheal stools of the latter; (2) in the blood of mice and fowls (S. gallinarum causing relapsing fever in fowls and Sp. anserina found in similar condition of geese); (3) in various ulcerative and gangrenous processes of man; in certain sub-acute and chronic pulmonary lesions.[1]

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[1] Deutsch. Med. Wchnschr., 1905, xxi, 1865; Berl. klin. Wchnschr., 1906, xliii, 185.

[2] New York State Jour. Med., 1907, vii, 177. (With good bibliography.)

[3] Jour. Inf. Dis., 1907, iv, 171.

The fowl spirochetes have been most studied. Marchoux and Salimbeni were the first to show that the tick, Argas persicus, is a carrier of these spirochetes. The mechanism whereby the tick infects the fowl has been minutely worked out by Nuttall[2] and his associates.

Noguchi has cultivated several species of spirochetes by the same method he used for growing Tr. pallidum (see below). Some of these he claims as new species, namely Tr. macrodentium and Tr. microdentium from the mouth, S. phagedensis from human genitals, Tr. calligyrum from condylomata and Tr. mucosum from pyorrhea alveolaris. Noguchi also reports the cultivation in successive transplants of Sp. recurrentis and Sp. duttoni.

King and Bauslack report finding a spirochete (S. hyos) in the blood of pigs suffering from hog cholera, which they consider the cause of the disease.

## TREPONEMA PALLIDUM (SPIROCHETA PALLIDA).

This organism is found in large numbers in *syphilis*, an infectious disease of human beings, characterized by its long course and by the definite stages of its clinical manifestations.

**Historical Note.**—Notwithstanding the fact that syphilis is one of the oldest diseases known and studied, only comparatively recently has definite light been thrown upon its cause in the discovery of the Treponema pallidum (Schaudinn and Hoffmann,[3] 1905).

Before this it was thought that the bacillus described by Lustgarten (1884) and others as occurring in small numbers in the lesions of syphilis bore an etiological relationship to the disease, but there were no evidences to support this view. Many other bacteria have been erroneously regarded as the probable cause of syphilis.

From time to time various observers have described protozoan-like bodies in syphilitic lesions, but their observations have not been confirmed.

Schaudinn announced early in 1905 that working with Hoffmann he found in the fresh exudates of chancre a spiral organism possessing characteristics similar to those of the spirochetes and he named it Spirocheta pallida. Later he concluded that this organism was individual enough (that is, it showed no undulating membrane, but possessed flagella) to be placed in a separate genus, so he called it Treponema pallidum. He thought that the organism was the cause of the disease. Since then there have been extensive studies on human syphilis and on experimental syphilis in lower animals with the result that the work of Schaudinn and Hoffmann has been abundantly corroborated and many new facts, given below, have been brought out.

**The Organism** (Fig. 178).—The Treponema pallidum is a very delicate structure closely resembling in morphology and staining reactions the *Treponema dentium*. It is somewhat longer, 4μ to 20μ long (average 10μ), and thinner, $\frac{1}{4}\mu$ to $\frac{1}{2}\mu$ in diameter. It has three to twenty sharp, deep spirals. The relationship between the length and the depth of the spirals is different in the two species; in *Treponema pallidum* length is to depth as 1 is to 1-1.5 ($1\mu$ long and $1\mu$ to $1.5\mu$ deep), while in *Treponema dentium* the average relationship is 1 to 0.5, the spirals being more shallow. The angle of the spiral turn is very sharp in both forms (more than 90°).

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[1] Dalimier: Presse Méd., 1919, xxvii, 124. Thompson: Brit. Med. Jour., 1918, ii, 709.

[2] Parasit., 1913, v, 262.

[3] Arbeit. a. d. Kaiserl. Gesundh., 1905, xxii.

Flagella-like anterior and posterior prolongations are often seen in the *pallidum*. The double flagella occurring rarely at one end are interpreted by Schaudinn as beginning longitudinal division. Schaudinn states that the division occurs very quickly (hence the reason why so few dividing forms are seen in stained preparations) and that it may be followed only by the most experienced observers during life. In the living condition the organism is not very refractive, so it is seen at first with difficulty. Its characteristic movements are rotation on its long axis, quivering movements up and down the spiral which is comparatively rigid, slight forward and backward motion and bending of the entire body. By the use of the ultramicroscope the motility of the organism is clearly seen (Fig. 180). Such a dark field examination is especially useful in the early diagnosis of an untreated uninfected lesion. A negative result is presumptive proof that the lesion is not syphilis.

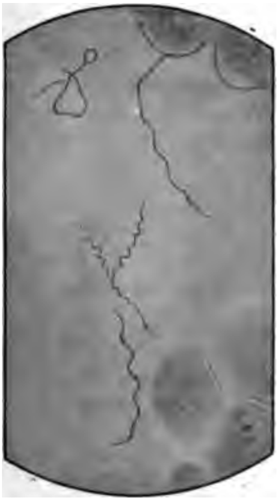

FIG. 179.—The two spirochetes in the center are Tr. pallidum; the three others, Sp. refringens. (Schaudinn and Hoffmann.)

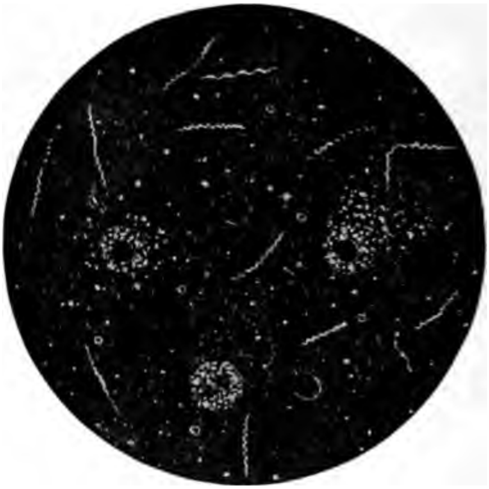

FIG. 180.—Treponema pallidum appearing as bright refractive body on a dark field as shown by India ink or ultramicroscope.

**Examination in Fixed Preparations.**—The staining methods that have given the most satisfactory results may be found in Chapter III. The spirochetes on the whole take all stains faintly, but they may be clearly demonstrated in smears by the India ink method (Fig. 180) and in sections by the silver impregnation method.

**Cultivation.**—In 1907 Levaditi and McIntosh[1] obtained impure cultures of spirochetes in collodion sacs containing human serum and syphilitic material and placed in the peritoneal cavity of a monkey (*Macacus cynomolgus*). Schereschewsky[2] reported that he had obtained impure cultures of a spirochete from syphilitic lesions and blood in the following culture medium: horse serum sterilized by heat (58° to 60° C.) until it is of jelly-like consistency, and afterward autolyzed at 37° for three days. A piece of tissue excised from the lesion (*e. g.*, base of a papule or part of a lymph node) is inoculated into this medium, and grown at 37° C. The culture begins in three days, but the optimum is reached in five to twelve days.

Mühlens (1909) and later Hoffmann (1911), reported that they had also obtained cultures of a pallidum-like spirochete from syphilitic lymph nodes, grown at first in Schereschewsky's medium and afterward transplanted to broth and grown anaërobically. Animal experiments are negative.

Levaditi and Stanesco about the same time reported growing two species of spirochetes from a case of balanitis. One, a new one, which they found very like pallidum, but non-pathogenic for monkeys, and which they named *S. gracilis;* the other *S. balanitidis.* They employed as media (1) collodion sacs in tubes of fluid horse serum; (2) horse or human serum heated to 75° C. These spirochetes were never obtained in pure culture.

Noguchi (1911) obtained pure cultures from syphilitic lesions in the rabbit, and later from human beings. His culture medium contained in deep tubes was a mixture of 1 part of ascitic or hydrocele fluid and 2 parts of 2 per cent. agar, in which was placed a small portion of sterile rabbit's kidney or other organ. The medium was covered by a deep layer of albolene. The spirochetes inoculated along a central stick grow out into the medium as a diffuse layer after ten days at 33.5° C., while most contaminating bacteria remain in the line of inoculation. Subcultures made away from the contaminating growths, finally become pure. Noguchi states that he has obtained syphilitic papules by scarifying monkeys (*cercopithecus* and *macacus*), and that such monkeys' blood gives a positive Wassermann reaction.

Zinsser and Hopkins[3] as well as others have corroborated this work of Noguchi. Gilbert found that later pure cultures of spirochetes grow abundantly when slant egg media are used instead of kidney tissue.

**Resistance of the Organism Outside the Body.**—Mixed cultures can live in different light and at room temperature for eleven and one-half hours, while drying kills them within one hour (Zinsser and Hopkins.)

**Pathogenesis.**—So far as is known, syphilis spontaneously appears only in man. Since 1879, when Klebs stated that he had produced syphilis in monkeys by the inoculation of human virus, various experimenters have reported its transmissibility to these animals by direct inoculation. Most of the earlier reports did not state the exact identity of the animals employed nor did they give details of methods and results.

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[1] Ann. de l'Inst. Past., 1907, xxi, 784.

[2] Centralbl. f. Bakt., 1908, xlv, 91.

[3] Jour. Exp. Med., 1915, xxi, 576; 1916, xxiii, 323, 341; xxiv, 561.

Metchnikoff and Roux, in 1903, produced a typical chancre on the genital mucosa of the young chimpanzee twenty-six days after inoculation. The essential lesion was followed by inguinal adenitis, and thirty days later by a generalized papular eruption. The virus was transferred in this case to lower monkeys. Most monkeys developed a primary lesion only, but some had abundant secondaries.

Since the discovery of the Tr. pallidum, experiments on monkeys have been more numerous and have been followed by more helpful results. More has been learned about the course of the infection in man, the evidence in favor of the Tr. pallidum being the cause of the disease has been strengthened, and many interesting investigations in regard to immunity have been made. Usually inoculations made by scarification on eyebrows or genitals are successful. The primary lesions are seen generally about thirty days after infection. In somewhat more than half the cases, after a slightly longer period, secondary symptoms (squamous papules on skin, and mucous patches in mouth) show themselves. No tertiary symptoms have been observed. Rabbits were first successfully inoculated by Hansell, then by Bertarelli.[1] The eye and the testes are favorable localities. Generalized syphilis has been produced in young rabbits by intracardiac inoculation (Uhlenhuth and Mulzer[2]).

Important features in regard to course of the infection have been summarized by Ewing[3] as follows: "If the virus is applied to the broken epithelium, a chancre develops, but if similar virus is inoculated into the subcutaneous tissue an initial lesion does not follow, immunity does not develop, and the animals remain susceptible to subsequent inoculation of the epithelium. Yet in several instances Neisser was unable to produce chancres in monkeys which had previously received subcutaneous injections of syphilitic material, indicating that immunity may sometimes appear after such subcutaneous injections. Possibly the leukocytes of the subcutaneous tissue destroy the virus before it can begin to multiply. Hence small superficial wounds may be more dangerous in man than deep ones. Nevertheless, it is recorded by Jullien that two French surgeons, accidentally inoculated by deep needle punctures, developed pronounced signs of constitutional syphilis, as attested by Fournier, but failed at any time to show signs of a chancre at the point of inoculation.

"In monkeys the virus exhibits a certain choice of epithelium for its entry. The abdominal skin resists the entry, the eyebrows and genitals are most readily inoculable in apes, and the palpebral borders in catharinians. The period of incubation varies from thirty days, on the average, in the chimpanzee, to twenty-three days in lower monkeys, but the shorter the incubation, the shorter and less severe the subsequent disease.

"That the virus circulates in the blood in certain stages of syphilis has been clearly shown experimentally. Although Neisser inoculated human subjects with the blood of florid syphilis without effect, a result which is now explicable, Hoffmann, in two of four experiments, produced syphilis in monkeys (Macacus rhesus) by inoculating the skin with human blood drawn forty days and six months after the appearance of the chancre. The resulting primary lesions were typical, appearing after the usual incubation and showing a characteristic histological structure and the presence of Tr. pallidum.

"Syphilographers are agreed that tertiary lesions are not contagious. Experimental studies have shown, however, that some tertiary lesions are capable of transmitting the disease. Salmon had negative results with an ulcerated gumma in the eighth year of the disease. Yet Neisser produced chancres and secondaries in a gibbon and in a macacus with the material from a non-ulcerated gumma

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[1] Centralbl. f. Bakt., 1906–1907, xli, 520, 639; xliii, 167, 328.

[2] Berl. klin. Wchnschr., 1910, xlvii, 1169.

[3] New York State Jour. Med., 1907, vii, 177.

(duration unknown), but the periods of incubation were very long, fifty-one and sixty-eight days. None of these observations invalidates the clinical experience that tertiary lesions are practically harmless for the patient's neighbors, but suggest greater caution in dealing with tertiary lesions.

"According to Colles's law, a mother who gives birth to a syphilitic infant may not herself contract the disease, but thereafter remains immune to inoculation. This law may be explained by the infection of the embryo or ovum, and the transference of immunity to the mother by the blood or by some other method. The probable mode of origin of the maternal immunity is suggested by an observation of Buschke and Fischer who found spirochetes in the inguinal lymph nodes of such a case which remained entirely free from the symptoms of the disease. Levaditi and Sauvage claim to have shown that Tr. pallidum is capable of invading the ovum. Finger and Landsteiner found the semen in one case of secondary lues, infectious for apes, but in other cases their results were negative. It is therefore only necessary to suppose an occasional escape from the genital tract in order to complete the necessary conditions for the infection of the embryo with immunity in the mother.

"Neisser endeavored to determine the degree and duration of the infectivity of the organs of monkeys and found that the virus persists especially in the blood-forming organs, spleen, lymph nodes, and marrow, while in the testicle also the virus is long preserved in active form. The other organs gave entirely negative results."

The virus is not filtrable, though the spirochetes can grow through the pores of Berkefeld filter V and N in about four days (Noguchi). It is readily destroyed by heat (52° C.).

*Luetin.*—Noguchi found that an extract from his cultures, which he calls luetin, gives a characteristic cutaneous reaction in syphilitic infection. In normal persons there is a slight local erythema, with possibly a small papule on the second day. In syphilitics there are three types of positive reaction:

1. Papular type. Large indurated red papule which increases for about four days.
2. Pustular type. The papule changes first into a vesicle and then into a pustule.
3. Torpid type. After a ten-day latent period a small papule forms which changes into a pustule.

These observations of Noguchi have been corroborated by Cohan and Robinson.

**Symptoms in Man.**—The course of the disease is divided into three stages: primary, secondary, and tertiary. The general character of the lesions in these stages is a more or less circumscribed formation of new tissue which is largely made up of small spheroidal cells alone or accompanied by a few endothelioid cells, and occasional giant cells.

The initial or primary lesion occurs in the form of a papule which develops into the so-called chancre, an ulcer with hardened base. Following this there is hyperplasia of the nearest lymph nodes. These lesions subside and six or seven weeks later the secondary lesions appear in various general eruptions on skin and mucous membranes and in other constitutional disturbances. The tertiary lesions which consist principally of the masses of new tissue called gummata are found throughout the viscera and in the periosteum.

Schaudinn's spirochetes have been demonstrated in all the lesions of syphilis (they are most easily demonstrated in the primary and secondary lesions), including the congenital types.

**Immunity.**—Natural immunity in syphilis is very peculiar. After the development of the primary lesions, man is usually insusceptible to reinoculation during the active stage of the disease, but during all stages both man and monkey can, in some cases, be reinoculated. Reinoculation in the tertiary stage gives precocious lesions of the tertiary type, gummata, and tubercles. Neisser found reinoculation from twenty-four to one hundred and four days after primary inoculation in monkeys sometimes effective, more often negative. During the stage when the skin is refractory to inoculation secondaries develop, showing that there is no complete immunity of the skin to the virus, since the *Treponema* is abundantly present in the lesions. Neisser suggests that cutaneous secondaries develop at periods of relative deficiency of immunity. He has shown that failure of reinoculation is not due to immunity to foreign infection and susceptibility to auto-infection, since the patient's own virus in both man and monkey is ineffective.

Many attempts to produce active immunity artificially have failed. Zinsser, Hopkins and McBurney[1] state that they can obtain from rabbits which have been inoculated with repeated doses of these culture spirochetes, sera with antibodies (agglutinin) for their non-virulent cultures of *Tr. pallidum* but not for the recently isolated virulent strains.

**Passive Immunization.**—Injection of large quantities of serum of syphilitics into chimpanzees has failed to produce definite immunity, neither has the serum of animals repeatedly inoculated with syphilitic virus or with pure cultures of the pallidum had any prophylactic effect against the virus.

**The Wassermann Reaction.**—Wassermann, Neisser, and Bruck were the first to apply the Bordet-Gengou phenomenon (see p. 255) to the diagnosis of syphilis. According to most workers, enough work has been done since then to establish its value as a diagnostic test. Some interesting points have been brought out in connection with this study, and many different methods have been recommended.

This test has been very widely used and positive results have been obtained in an immense number of cases (over 90 per cent. of cases with active general infection). In general paralysis and in the majority of cases of tabes a positive reaction occurs which may be given also by the cerebrospinal fluid. On account of the difficult technic involved it can only be of use in the hands of experienced workers. Positive results have been reported in a number of other diseases as well as syphilis, but in many cases these results have not been generally accepted, and in other cases the diseases showing positive reaction as yaws, leprosy, dourine, etc., have been, as a rule, confined to the tropical countries, or else the positive reaction has been found only during a limited stage of the disease, as in scarlet fever, or the differential diagnosis is otherwise marked, as with tuberculosis. The general opinion at the present time is that the test is of great practical value in the majority of cases of syphilis. It is used as a routine public health measure in New York City and several other centers. The reaction is practically always present during the secondary stage of the disease. The reaction gradually disappears when the disease becomes inactive or is cured. It may disappear before a cure is established, to reappear if an active process starts. Mercury treatment diminishes or annuls the reaction with an occasional intensification after a few treatments; while treatment with salvarsan may at first intensify it or make a negative reaction positive. A good report of the diagnosis and treatment of syphilis is given by Fordyce.[1]

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[1] Jour. Exp. Med., 1915, xxi, 576; 1916, xxiii, 323, 341; xxiv, 561.

The nature of the reaction and the technic of the test are considered in Part I, p. 255.

**Colloidal Gold Test.**—In 1912 Lange[2] applied to spinal fluids the principle brought out by Zsigmondy (1901) that solutions of either electrolytes or of proteins precipitate colloidal gold, but that when both electrolytes and proteins are present the gold is not precipitated. Miller and his co-workers[3] have been able to standardize the reagent, so the test has become more reliable.

The test is simply another aid in helping to detect certain central nervous system involvements not responding so specifically to other tests. The test consists of a series of color changes in graded dilutions of spinal fluid, varying from negative salmon-red, then red-blue, blue, blue-gray, to colorless. Miller has worked out a "paretic curve."

**Spirochetes in Frambesia Tropica (Yaws).**—Castellani,[4] in 1906, announced that he had found in yaws a spiral organism which is called *Treponema pertenue*. He determined that monkeys (*macacus, semnopithecus*) are susceptible to inoculations with material from yaws patients apparently containing only this spirochete. Such material filtered is inert. He states that monkeys successfully inoculated with yaws do not become immune for syphilis, neither do those having had syphilis become immune for yaws. Castellani further states that specific characteristics between the two diseases are also brought out by means of the Wassermann reaction. His work has been corroborated by several observers. Frambesia lesions similar to those produced by syphilis in the testicles of rabbits have been obtained by Nichols.[5] Levaditi and Nallan-Larrier[6] state that monkeys infected with syphilis are refractory to yaws, while those infected with yaws are susceptible to syphilis; therefore they conclude that yaws is a mild form of syphilis. The treatment of this condition by Castellani's mixture has given good results.[7]

**Spirochetes in Infectious Jaundice.**—Inada, Ido[8] and their collaborators in Japan, and Heubner and Reiter in Germany have reported the presence of spirochetes (*S. icterohemorrhagiæ*) in infectious jaundice or

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[1] Trans. of Congress of Phys. and Surg., 1917, New Haven, Conn.

[2] Berl. klin. Wchnschr., 1912, xlix, 897; Ztschr. f. Chem., 1913, i, 44.

[3] Bull. Johns Hopkins Hosp., 1914, xxv, 123; 1915, xxvi, 391.

[4] Jour. Hygiene, 1907, vii, 558.

[5] Jour. Exp. Med., 1910, xii, 616; 1911, xiv, 196.

[6] Ann. de l'Inst. Past., 1908, xxii, 260.

[7] Phila. Jour. Sc., 1918, Sec. D, p. 89.

[8] Jour. Exp. Med., 1916, xxiii, 377, 557; xxiv, 471.

Weil's disease. This work was confirmed by Stokes and by Noguchi. Ido and his co-workers claim to have found the same species in both house and field rats, and to have obtained active immunity in guinea-pigs against the organism. Noguchi[1] considers this a new genus and has suggested the name *Leptospira* for it. He reports finding the same species in the rats captured in New York City and vicinity. Jobling and Eggstein[2] report finding a similar spirochete in the wild rats of the Southern States and Otteraaen[3] found a similar spirochete in the rats about Chicago. Inada and others[4] recommend intravenous serotherapy.

**Spirochetes in Rat-bite Fever.**—Futaki and his associates have reported the presence of a spirochete similar to *S. recurrentis* in cases of rat-bite fever in Japan. (See p. 529 for streptothricis in rat-bite fever.)

## SPIRONEMA OBERMEIERI (S. RECURRENTIS) AND ALLIES.

The organisms in this group are classed with the spiochetes as protozoa by Schaudinn, Hartmann, Mühlens, and others, but by Norris, Novy, and others they are still placed with the bacteria. Novy and Knapp[5] have made extensive studies of *S. obermeieri* (the cause of relapsing fever in Europe) as well as of *S. duttoni* (the cause of tick fever), spirochetes from American relapsing fever, and *S. gallinarum* (fowl spirochete) and consider that they have demonstrated their bacterial nature and that many, if not all, spirochetes should be placed in this group.

**Spironema (spirillum) obermeieri** was first observed by Obermeier in 1873 in the blood of persons suffering from relapsing fever. It was found in large numbers during the height of the fever, it disappeared about the time of the crisis, and reappeared during the relapses. It was not found in other diseases. Obermeier considered it the cause of the disease, and his views were shown to be correct by the production of the disease in man and ape through experimental inoculation.

**Morphology.**—The organisms are long, slender, flexible, spiral or wavy filaments, with pointed ends, from 16μ to 40μ in length and from one-quarter to one-third the thickness of the cholera spirillum (2½μ to ¾μ). Novy has demonstrated a terminal flagellum (Fig. 181). They possess three to twelve wide, more or less irregular spirals. They stain somewhat faintly with watery solutions of the basic anilin dyes, better with Löffler's or Kühne's methylene-blue solutions, or with carbol fuchsin; best with the Romanowsky method or its modifications. They are negative to Gram.

**Biological Characteristics.**—In fresh preparations from the blood the spirochetes exhibit active progressive movements, accompanied by very rapid rotation in the long axis of the spiral filaments or by undulating movements. They are found only in the blood or blood organs, never in the secretions, and only during the fever, not in the intermissions, or at most singly at the beginning of, or for a short time, after, an attack.

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[1] Jour. Exp. Med., 1917, xxv, 755, and 1918, xxvii, 575.

[2] Jour. Am. Med. Assn., 1917, lxix, 1787.

[3] Jour. Inf. Dis., 1919, xxiv, 485.

[4] Boll. dell. Ist. Sier., 1917, i-iii.

[5] Jour. Inf. Dis., 1906, iii, 291.

When kept in blood serum, or a 0.6 per cent. solution of sodium chloride, they continue to exhibit active movements for a considerable time. They may be preserved alive and active for many days in sealed tubes. They are killed quickly at 60° C., but they remain alive for some time at 0° C. Unsuccessful efforts to cultivate them in artificial culture media have been made from time to time. Koch has observed an increase in the length of the spirilla and the formation of a tangled mass of filaments. Novy finally succeeded in cultivating them in celloidin capsules placed in the peritoneum of rats. His culture remained virulent (with a slight loss) for many generations. Noguchi has also cultured them.

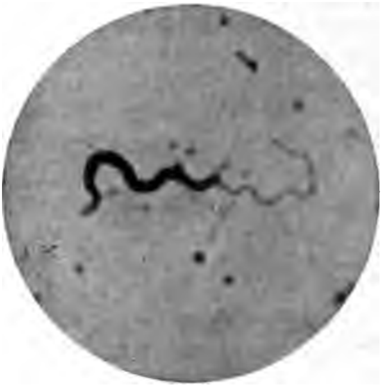

FIG. 181.—Photograph of Sp. obermeieri showing terminal flagellum. × 3000 diameters. (After Novy.)

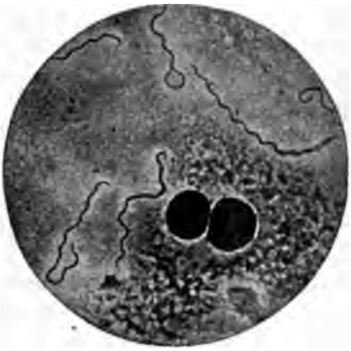

FIG. 182.—Spirocheta obermeieri blood-smear. Fuchsin. × 1000 diameters. (From Itzerott and Niemann.)

**Pathogenesis.**—In man, whether the disease is acquired naturally or by artificial inoculation, the organism causes the following symptoms: After a short period of incubation the temperature rises rapidly, remains high for five to seven days, and then returns to normal by crisis. About seven days later there is another sudden rise of temperature, but this time the crisis occurs sooner. A second or third relapse may occur. The organisms increase in numbers rapidly in the blood from the beginning of the fever, large numbers often being found in every microscopic field. They begin to disappear a short time before the crisis, and immediately after the crisis it is practically impossible to find them in the circulating blood. The mortality varies in different epidemics from 2 to 10 per cent. When monkeys are inoculated with human blood containing the spirilla, they become sick about three and a half days later, but show only the initial febrile attack or, at the most, an occasional short relapse. The organisms are found to have the same relation to the pyrexial periods as in man. Blood from one animal taken during the fever induces a similar febrile paroxysm when inoculated into another animal.

Metchnikoff showed that during the intermissions when the spirochetes disappeared from the circulating blood they accumulated in the spleen and were ingested in large numbers by certain phagocytes and finally were destroyed.

According to Lamb, a certain amount of immunity is conferred upon monkeys (*Macacus radiatus*) soon after an attack, but it disappears quickly. If the serum is removed during this time it is found to have some protective action when mixed with the blood containing spirilla and also to cause agglutination of the organisms. Novy (1906) showed that a powerful specific germicidal body exists in the blood of rats during and after recovery, notably in the blood of hyperimmunized rats. An immunizing body probably distinct from this is also present. He also showed that passive immunity can be imparted by injections of recovered or hyperimmunized blood, that both active and passive immunity may last for months, and that the serum has both a preventive and a curative action. Agglutinins are also present in such a serum.

Breinl has shown that the immunity produced by *S. obermeieri* does not protect against *S. duttoni*, and *vice versa*. Neither does it protect against the Asiatic or American variety of this type of spirochete.

The strain found in Bombay seems to be more virulent than that in Europe.

**Spironema Duttoni.**—The organism shown by Dutton (1905) to be the cause of African tick fever is very similar morphologically to *S. obermeieri*, but Novy, Fränkel, and others have shown slight differences which make them believe that it is another variety, if not another species of this group.

**Spironema Carteri.**—This spirochete was described by Carter in 1877 as causing relapsing fever in Bombay. Monkeys were inoculated by Carter successfully with the human blood containing this spirochete.

**Spirochetes from Relapsing Fever in America.**—Darling[1] has reported a study of the relapsing fever in Panama. He isolated the organisms in two cases and studied their characteristics. He finds they agree with those reported by Carlisle, Norris, and Novy for the organisms isolated by Norris,[2] but they can only be differentiated from the other relapsing fever spirochetes by animal inoculations and by the disease in humans Moreover, he finds that in all probability a polyvalent serum may be necessary for cure, since the serum from one strain did not protect against the other strain.

**Filtrability of Spironema.**—A number of workers have demonstrated that spironema may pass through Berkefeld's N and V under pressure.

## INSECT CARRIERS OF SPIROCHETES AND ALLIES.

Each species of spironema is conveyed by the bite of a specific arthropod. Many studies have been made on this subject since the work of

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[1] Arch. Int. Med., 1909, iv, 150.

[2] Jour. Inf. Dis., 1906, p. 527.

Marchoux and Salimbeni on fowl spirochetes, and of Dutton and Todd on African tick fever. Nuttall and his associates have added much to our knowledge of ticks and other arthropods as vectors. Dutton and Todd demonstrated that *S. duttoni* can be transferred to monkeys by the bites of young ticks (*Ornithodorus moubata*) at their first feed after hatching from infected parents. He accidentally demonstrated the fact that the disease can be inoculated into human beings through a cut surface, for after a wound received at autopsy he developed the disease which eventually caused his death.

Koch corroborated these authors' results. Leishman[1] demonstrated that the second generation of ticks may also infect. He was unable to find spirochetes in tissues of ticks later than ten days after feeding, though young ticks from eggs hatched after this time were infective. He found, however, granules which he considers changed spirochetes. Hindle[2] agrees with him. The fact that ticks may be infective long after the disappearance of typical spirochetes, if they are placed at 30° to 35° C. is evidence of the infectious nature of these granules. Möller found that a tick may remain infective for a year and a half or more after its initial feed from an infected host. Möller also showed that the third generation of ticks might inherit infection. Sargeant and Foley and later Balfour have observed another type of relapsing fever in Africa, which they consider due to a different variety of spirochete (*S. berbera*). Mackie obtained strong evidence that the body louse is the chief carrier of *S. recurrentis*. His observations have been corroborated by a number of observers.

Nicolle and his colleagues found after twenty-four hours spirochetes disappear from gut of the louse (*P. vestimenti*) to reappear in from eight to twelve days and continue for eleven days and possibly longer. Monkeys inoculated with the contents of lice fifteen days after feeding develop relapsing fever.

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[1] Jour. Royal Army Med. Corps, 1909, xii, 123: Lancet, 1910, i, 1.

[2] Parasitology, 1911, iv, 133 and 183.

# CHAPTER XL.

# BODO. POLYMASTIGIDA.

## BODO LACERTÆ (GRASSI).

Bodo lacertæ is frequently found in the intestinal contents of most of the higher animals, hence it is easily obtained for class study. A species of the *Bodo* has been observed in human urine (***Bodo urinarius***), but it is probably a harmless invader.

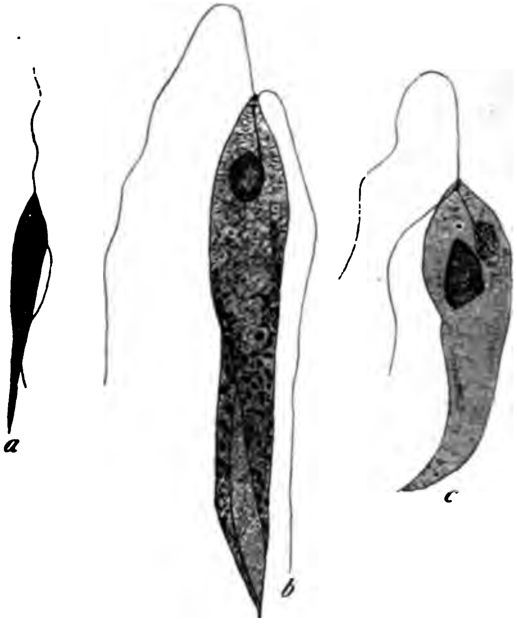

FIG. 183.—Bodo lacertæ (Grassi): *a*, living; *b* and *c* stained; *b* type without, *c* type with chromidium (1000 : 1). (After v. Prowazek, from Kisskalt and Hartmann.)

*Bodo lacertæ* is wedge-shaped, the posterior part of the body being turned a half to a whole spiral on itself. It possesses two characteristic flagella, equal in thickness but unequal in length. In motion the longer one is directed forward, while the shorter is carried backward, functioning as a rudder, or a towing flagellum. Both flagella spring from a basal granule which are well demonstrated by the iron hematoxylin stain. They are situated in the extreme anterior part of the body and are attached to the nucleus by a delicate fibril (Fig. 183, *b*, *c*, and Plate IV, Figs. 1, 3). The movement of the organism is characteristic, it consists in a rapid irregular swimming in various directions with the anterior flagellum moving from side to side. The body itself shows a slightly sinuous motion.

There are two types of nuclei seen. First, the typical vesicular nucleus most frequently seen among the flagellates. This is round and has a definite membrane about which chromatin is arranged in irregular masses. In the center, or eccentrically placed, is a compact karyosome. Iron hematoxylin preparations bring out an achromatic network between the chromatin masses and the karyosome. In the living condition the nucleus appears as a greenish glistening refractive vesicle. The second type of nuclear apparatus is seen in smaller organisms. This is a similar nucleus except that it is smaller and more compact; posterior to this is another nuclear-like body, varying much in shape and arrangement of chromatin (Fig. 183, *c*). This is the sexual chromidia.

The cytoplasm appears in iron hematoxylin stained specimens as finely reticular. It contains many deeply stained granules. There is no mouth opening. Food is taken in by osmosis.

In propagation, the two types just described develop differently. The first or ordinary type forms around division cysts. The flagella disappear and a delicate cyst membrane is formed. The increase in the size of the nucleus and the subsequent division may be followed in life. It lasts about twenty minutes. After a single or, more seldom, a double division of the cell, the daughter cells, while still within the cyst, form their flagella, become very motile, finally break the cyst wall and swim out. The second type reproduces, in the free living condition by longitudinal division. The basal granules divide, the principal nucleus divides by mitotic division, the chromidia by amitosis. The whole process can be seen in hematoxylin preparations. Sexual division in this species occurs in cysts by autogamy. It is not easily followed in life because of the high refraction of the cyst. The changes must therefore be studied in specimens stained with iron hematoxylin.

They are briefly as follows: The nucleus becomes larger and about its membrane appear small spheres of chromatin which finally leave the nucleus and gather together, forming the so-called chromidial or sexual nucleus, while the original or somatic nucleus gradually degenerates. The new nucleus divides amitotically into two daughter nuclei; from these two, smaller parts are then separated, as reduction nuclei, which also degenerate. The remaining parts of the two nuclei increase in size and then fuse to form a new nucleus. The organism may then leave the cyst or the cyst may become a lasting cyst and serve to infect a new host.

Besides this method of fructification by autogamy in a cyst, is seen, though seldom, a copulation between two individuals of different sizes which afterward become encysted and divide into two to sixteen daughter flagellates.

## POLYMASTIGIDA.

The order polymastigida consists of flagellates having several flagella projecting from different parts of the body. The majority of the forms known are parasitic in certain fish. Recently more reports are coming from the tropics of the possibility of these forms being pathogenic for man.[1]

**Trichomonas Vaginalis.**—Donné, in 1837, described a form which he found in the human vagina, and which he therefore called Trichomonas vaginalis. It has been found by other observers to be a frequent habitant of the vagina at all ages. It is also found occasionally in the acid urine of both sexes. The mode of infection of the female is unknown. The body of the parasite at rest is pear-shaped, but during action its ameboid movements cause it to assume various shapes. The size varies from 12μ to 25μ long and 8μ to 15μ wide. The protoplasm is finely granular, excepting for two rows of larger granules which begin on either side of the nucleus and converge posteriorly. From the anterior part project three or four flagella, which seem to begin at a basal thickening near to, or connected with, the more or less oval, indistinctly vesicular nucleus. From the origin of the flagella an undulating membrane extends backward. The body also seems to possess a certain linear structure connected with the membrane. Contractile vacuoles have not been seen.

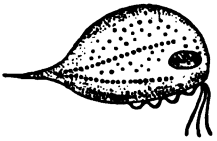

FIG. 184.—Trichomonas vaginalis. (Blochmann.)

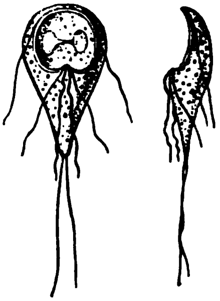

FIG. 185.—Lamblia intestinalis. (Schewiakoff.)

**Trichomonas Hominis Davaine.**—This form, found frequently in the human alimentary canal, is very similar to the Trichomonas vaginalis, but it is smaller and more pear-shaped. It has been found often in acute diarrheas, and the causal relation between it and the pathological process has been accepted, and since the war many cases of trichomoniasis have been reported.

A similar form has been seen a few times in lung gangrene, aspiration pneumonia, and bronchiectasis.

**Lamblia intestinalis** (Lambl, 1859), a flagellate belonging to this group, parasitic in the small intestines of mice, rats, rabbits, dogs, cats, and sheep, has also been found occasionally in the human intestines. It is beet-shaped, bilaterally symmetrical, 10μ to 21μ long and 5μ to 12μ wide, possessing flagella 9μ to 14μ long. Anteriorly, this species has a characteristic concavity, the rim of which seems to be contractile, forming a sucking apparatus. The eight flagella of the organism are arranged in pairs: one anteriorly, two laterally, and one posteriorly. The nucleus is situated anteriorly and has a central constriction. The protoplasm of the body is thick and hyaline. Contractile vacuoles have not been seen. Schaudinn (1906) observed encystment, copulation, and complicated nuclear changes in this organism.

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[1] Haughwout: Philippine Jour. of Sc., 1918, xiii, 217.

Infection follows the ingestion of the cysts with unclean food. The parasites fasten themselves to the free surfaces of the epithelial cells by their sucking apparatus, but seem to exert little harmful influence on their hosts. They have been found most frequently in poor children who play often in dirt containing the cysts. A number of adult cases of apparent lambiosis have been reported recently by Labbé and others.[1] Repeated small doses of calomel will usually cause their disappearance from the feces.

[1] Labbé: Presse Méd., 1919, xxvii, 161.

# CHAPTER XLI.

## AMEBA.

SEVERAL authors have reported the finding of amebæ in man, especially in so-called tropical, ulcerative, or amebic dysentery, but as the first descriptions were incomplete and the laws of nomenclature were not strictly followed there resulted many synonyms for the same species and many species bearing the same name.

At present the four that were considered as distinct species have now become three according to some authors, and only two of those described, from a practical standpoint, need be considered. These are Entameba histolytica, the form described by Schaudinn[1] from tropical dysentery and considered by him the cause of that disease, and Entameba coli, the kind found in normal human intestines by Schaudinn and thought by him to be harmless.

The discussion in regard to the "parasitic" and the "non-parasitic" forms is still going on. Some investigators say that certain amebæ are strict parasites of the intestinal tract of mammals; that is, that these amebæ cannot be cultivated on artificial culture media, and that they are so different from the "saprophytic" forms, *i. e.*, those that are cultivated (?), that they must constitute a different genus.

Williams,[2] Calkins,[3] and others, on the contrary, believe that not enough study on the "cultural forms" has been done to draw definite conclusions as to their relationship to those seen in stained preparations from amebic dysentery.

**Historical Note.**—Stiles[4] has given a detailed history of the generic name Ameba and of the specific one Ameba proteus, and, finally, of the naming of the intestinal amebæ. He shows why the name Entameba should be given to the genus described by Lambl and Lösch.

This article illustrates very forcibly the absurdity of bringing forth new names for organisms only half studied and of claiming that such organisms belong to new genera.

The first report on intestinal amebæ of man was made by Lambl, in 1860, who announced the presence of ameboid forms in the intestinal mucus of a child who had died from enteritis. Supposedly the same forms were more fully described by Lösch, in 1875, under the name Ameba coli. Lösch found his organisms in stools of a patient suffering from chronic dysentery and he succeeded by rectal injections in producing superficial ulceration in the large intestines of dogs. He therefore claimed that this organism is the cause of dysentery. His work was corroborated by many observers. In the meantimes amebæ were found in diseases other than dysentery, and Grassi, in 1879, reported them in healthy intestines. The work of Kartulis[5] (1886), however, helped largely to establish the fact that amebæ play an important part in the etiology of dysentery in Egypt. He was the first to find the organism in abscess of the liver in tropical dysentery. In our own country among the most important workers in this field are Councilman and Lafleur[1] (1891). They conclude that amebic dysentery should be regarded etiologically, clinically, and anatomically as a distinct disease. They disapprove, however, of the name Ameba coli and propose the name Ameba dysenteriæ for the pathogenic form; but as they do not show in any way than by its pathogenesis that the species they describe is a new one, their name, according to the rules of zoölogical nomenclature, cannot be accepted. Harris's[2] work, too, is important in showing an etiological relationship between amebæ and a certain form of dysentery, but neither did he describe the morphology of his organism minutely enough to identify it with Schaudinn's histolytica which is described below. Casagrandi and Barbagallo, in 1897, were the first to claim that the amebæ so far described in man show differences enough from the fresh-water amebæ to belong to a new genus. They therefore created the genus Entameba and gave the specific name Entameba hominis to amebæ of the Ameba coli type. Schaudinn and Stiles agree with them as to the generic name, but consider that the correct specific name is complicated by the fact that there are different species in this group. Many observers (Kartulis, Councilman and Lafleur, Quincke and Roos, Kruse and Pasquale) have considered that there are different varieties in the human intestines, but they have given no morphological differences distinct enough to classify such varieties. Schaudinn is the first who bases upon a definite morphology his claim (1903) that at least one species among them is pathogenic and one non-pathogenic. The latter, which he found in normal human intestines, he says resembles those already described as Ameba coli; therefore he gives it the name Entameba coli; while the former, which he found exclusively in ulcerative tropical dysentery, he calls Entameba histolytica.

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[1] Arbeit. a. d. Kaiserl. Gesundh.-amte, 1903, xix, 547.

[2] Jour. Med. Res., 1911, xx, 263.

[3] Protozoölogy, Lea & Febiger, Philadelphia, 1909.

[4] Am. Public Health Assn., 1904.

[5] Kolle and Wassermann, 1913, 2d ed.

The different views upon the relationship to disease of amebæ found in the human intestines may be summarized as follows:

1. That the amebæ in man have no pathogenic properties, hence are not the cause of amebic dysentery. (Cunningham, Grassi, Celli and Fiocca, Casagrandi and Barbagallo, and others.)
2. That any intestinal ameba may become pathogenic and cause the specific malady known as amebic dysentery. (Musgrave, Clegg, and others.)
3. That amebæ are able to keep up a preëxisting inflammation. This was the original view advanced by Lösch when he described the most commonly cited form, Ameba coli, and several authors have followed Lösch in this opinion.
4. That more than one species of amebæ are found in man, at least one pathogenic, and one non-pathogenic. (Kartulis, Councilman and Lafleur, Quincke and Roos, Strong, Schaudinn, Craig, and others.)

The study of bacillary dysentery by Shiga, Kruse, Flexner, and others (see under Bacillary Dysentery) has demonstrated that there are at least two forms of dysentery, one produced by amebæ and the other by bacilli, and from the work on the former it now seems certain that it is produced by one or more specific forms of amebæ.

Amebæ have been reported in teeth cement and in carious teeth as well as in abscesses of the jaw. Flexner, in 1892, described an amebic organism in the latter condition, and considered it identical with the organism described by Lösch, Councilman and Lafleur as Ameba coli. In the same year Kartulis described similar organisms found in similar lesions. Gross and Sternberg found them in tartar of teeth. Smith, Barrett, Bass and others have recently found amebæ in practically all cases of Rigg's disease and have claimed an etiological relationship which many other authors have combated. Prowazek (1904) described a mouth ameba as a distinct morphological species under the name of Entameba buccalis.

Successful cultures have been made of amebæ obtained from the intestines of man and other animals, as well as from certain fruits and vegetables.

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[1] Johns Hopkins Hosp. Rep., 1891, ii, 395.

[2] Am. Jour. Med. Sc., 1905.

Musgrave and Clegg[1] (1904) studied amebæ in the Philippines by the culture method and came to the conclusion that forms obtained from various sources were probably all a single species.

Williams[2] (1911) obtained pure cultures on sterile "tissue media" of amebæ isolated from the intestines of mammals, including one from a case of amebic dysentery.

**Sites of Amebæ in the Human Body.**—Intestines and neighboring tissues; abdominal cavity; abscess of liver, lung, pleura, brain, and mouth; necrosis of jaw-bone; tartar of teeth; urine.

**Materials and Methods for Study.**—Human cases of dysentery showing amebæ in stools are so seldom on hand in the northern part of this country that they cannot be counted on. The non-pathogenic form in human intestines might be obtained after administration of a saline cathartic, but generally one must depend upon saprophytic forms for work with students, or upon cultures obtained from cases of amebic dysentery. Material rich in saprophytic forms may be obtained from an infusion in water of lettuce, cabbage, potato skins, or other vegetable material. Such an infusion should be made a week or two before it is needed, when it will be found that the pellicle which forms contains many varieties of protozoa and bacteria, among which are generally large numbers of ameboid forms. Often one may get good material from the feces of many of the lower animals, such as the lizard, toad, or guinea-pig.

If one has material containing human intestinal amebæ, kittens or puppies may be fed with the cysts in order to obtain a new supply. The amebæ should be examined in both the fresh and fixed condition. Cultures may also be made as described below.

**Examination of the Fresh Material.**—The study of the living amebæ is extremely important. This may be done by making a hanging drop or hanging mass (p. 73) from fluid containing amebæ. The size, kind of motion, frequency of pulsation of contractile vacuole, and as much of the cell contents as possible should be noted.

The stools should be examined on the warm stage as soon as possible after their passage (not later than two hours), and should be kept at blood heat until examined. A platinum loopful of material should be taken from the slimy masses in the thinner part of the feces, diluted with physiological salt solution, covered with a cover-glass, and examined under moderate magnification.

Harris[3] found that a drop of a watery solution of toluidine blue added to a small particle of the feces stains the entoplasm of the amebæ at once and the ectoplasm a few minutes later. The amebæ seem to be quickly killed and often when natural forms are beautifully preserved the preparations, after being washed in water and mounted in Farrant's medium, may be preserved for months, but after a time the stain completely fades.

**Permanent Preparations.**—Thin films are made on glass slides or cover-glasses, and immediately, before they are allowed to dry, they are placed in the fixing solution. Cover-glass may float film down, on the surface of the fixative. Among the best fixatives are: Hot sublimate alcohol (50° C.), Zenker's fluid, hot Hermann's fluid, or methyl alcohol (p. 74.)

**Stains.**—Several of the many good staining methods are given in the chapter on Stains.

1. Thin Delafield's hematoxylin, from one-half hour to several hours, then washed in water. (If overstained, the preparation may be differentiated in acid alcohol, controlling under the microscope, then washed in water.) The film or section is then passed successively through 70 to 95 per cent. and 100 per cent. alcohol, absolute alcohol + xylol, xylol, cedar oil, or Canada balsam.

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[1] Amebas: Their Cultivation and Etiological Significance, Manila Bureau of Public Printing, 1904.

[2] Jour. Med. Res., 1911, xx, 263.

[3] Am. Jour. Med. Sc., 1905.

2. Heidenhain's iron hematoxylin (see p. 84). The smear is changed from distilled water into the iron-alum mordant for four to twelve hours, or overnight; well washed in distilled water; in stain from two to twenty-four hours, excess washed out in the iron mordant, controlled under the microscope (as decolorization occurs very quickly) until the nucleus is sharply differentiated; the chromatin of the nucleus must be a deep blue black, and the cytoplasm a light gray; then a thorough washing in tap water and passage through the alcohols and xylol, and in Canada balsam, or cedar oil for mounting.

3. After fixation in methyl alcohol one may use Giemsa's staining method (see p. 83), or a modification of the method suggested by Van Gieson for staining the Negri bodies in smears (see p. 86).

Masses containing amebæ, as mucous flakes or portions of the intestinal or liver abscess wall in amebic dysentery, or pieces of decaying vegetables may be fixed *in toto* in hot sublimate alcohol for one-half hour, washed in iodin alcohol for twenty-four hours, passed through the different strength alcohols and imbedded in paraffin (see p. 87) for section cutting if desired.

**Cultures of Amebæ.**—Pure mixed cultures may be made in the following way: From the material containing amebæ a small loopful is removed with a platinum wire and isolated spots are touched over the surface of ameba agar (see p. 132) poured into sterile Petri dishes. If necessary, feces may be thinned with physiological salt solution before planting. In one to several days at 25° C. the amebæ with the accompanying bacteria may overgrow the entire plate. We have found that amebæ will grow as well upon nutrient agar—better with certain bacteria—as on the special medium just mentioned. Impression films may be made of these cultures, or small pieces of agar and culture may be imbedded entire. From such a culture the "pure mixed" cultures of Frosch may be made as follows: The amebæ which have crept out to the periphery of the growth are taken out with their accompanying bacteria and transplanted. Usually one or two organisms favorable for the growth of the amebæ accompany them and in this way one may finally get the amebæ growing with one definite bacterium. We have isolated from a culture a single ameba unaccompanied by bacteria by the following simple method: Under the low-power lens with a fine platinum loop an isolated ameba is drawn toward the edge of the agar plate. When it is well separated a disk of agar containing it is cut out following the margin of the objective and is transferred to a fresh agar plate. A very small quantity of a desired bacterium is now added to the disk near the ameba, and a "pure mixed" culture results.

**Pure Cultures.**—Certain varieties of amebæ from the intestines of mammals grow without other organisms, *i. e.*, grow in pure cultures, when inoculated on a piece of fresh sterile brain, kidney or liver placed upon Musgrave and Clegg's ameba agar or on nutrient agar. They grow abundantly on such media at temperatures varying from 22° C. to 38° C. (Williams).[1]

**Morphology.**—The morphological characteristics of the ameboid stage, as described by various observers, seem not to have been minutely enough studied to be depended upon in differentiating the species. Moreover, descriptions have differed markedly. While Schaudinn and others, especially Craig of the more recent writers, say that it is easy to differentiate between the ameboid forms of histolytica and coli, Musgrave and Clegg, Strong, Williams, Knowles and Cole[2] and others say the points of difference are not marked.

The observations of Schaudinn and others may be summarized as follows: (1) Ent. coli is, on the whole, smaller than Ent. histolytica;

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[1] Jour. Med. Res., 1911, xxv, 263.

[2] Ind. Jour. Med. Res., 1917, 498.

(2) its ectoplasm is so small in amount and so slightly differentiated that it is only seen when the organism puts forth pseudopods, while the cortical zone of the Ent. histolytica is wider and is distinctly differentiated from the entoplasm; (3) the pseudopods of the former are small, rounded, delicate, and not highly refractive, those of the latter are larger, finger-shaped, firmer, and more highly refractive, thus indicating the power of the organism to penetrate its host's tissues; (4) the nucleus of Ent. coli is very distinct in life as well as in stained spreads, due to a definite membrane, a more distinct karyosome, and much chromatin which is distributed throughout the nucleus with more of a collection about the periphery; the nucleus of Ent. histolytica on the other hand, is seen with difficulty during life, and stains faintly, owing to its delicate membrane, its small amount of chromatin, and small karyosome; the chromatin is collected about the karyosome and the periphery of the nucleus; the nucleus, moreover, is much more variable in shape, in the active organism than is that of the Ent. coli; (5) the entoplasm of Ent. coli is less granular and vacuolated and contains fewer red blood cells than that of Ent. hystolytica which sometimes shows immense numbers of these blood cells (Plate IV, Fig. II, 1–5).

The above points of difference cited for organisms in the ameboid stage may hold for forms living in the human intestines; but we have found that organisms from widely different sources (*e. g.*, intestines of guinea-pigs and of dogs from New York and of humans from the Philippines) when grown with a favorable bacterium in the thermostat at body temperature may show appearances similar to each other and similar also to those described by Schaudinn for Ent. histolytica. More corroborative work, therefore, seems to be needed before we accept the above observations as being the whole truth. Varying reports still continue to come in regard to the differentiation of types of amebas in human stools.[1]

**Reproduction.**—In the vegetative stage probably all these forms divide by a primitive mitosis, though Schaudinn, Craig,[2] and others saw only amitosis. All of our culture forms divide by mitosis, and many observers have recently reported similar division in related forms. Schaudinn and others state that Ent. coli in the vegetative stage may divide by breaking up (schizogony) into, at the most, eight daughter cells. In the latter instance, according to these authors, the nucleus undergoes a somewhat complicated process of division. But recently Walker, Hartmann, and Williams have presented evidence to show that this is probably not true. The nuclei continue to divide by binary mitosis.

The vegetative stage of each intestinal organism takes place in the upper part of the intestines; as the feces become thicker most of the vegetative forms die off, while some pass on to permanent cyst formation. As with many coccidia, parasitic amebæ may pass through a long period of vegetative life before entering upon another phase wherein forms are produced capable of infecting a new host. The length of this period depends upon a number of circumstances. Under conditions favorable for the growth of the amebæ, as in cases of diarrhea, the vegetative phase is considerably lengthened, while in healthy intestines, as the amebæ pass down with the thickening feces, the infecting cysts are more or less quickly formed.

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[1] Smith: Ann. of Trop. Med. and Parasit., 1919, xiii, 1.

[2] Jour. Inf. Dis., 1908, v, 324; Arch. Int. Med., 1914, xiii, 917; Jour. Inf. Dis., 1913, xiii, 30.

The statement made by Schaudinn that Ent. histolytica during the vegetative stage may multiply by budding as well as by binary fission, is now considered incorrect. Unequal division, however, probably frequently occurs.

It is now thought by Walker, Hartmann, Craig, and others that Ent. tetragena, described by Viereck, Hartmann, and Craig as a distinct species, is merely a phase of Ent. histolytica, though Hartmann calls the species tetragena and drops the name histolytica.

The observations of Schaudinn on the sexual phenomena of Entamebæ, though earlier corroborated by Hartmann, Craig, and others, are now considered incorrect by these same observers. The subject awaits further investigation.

**Viability.**—The pathogenic amebæ are apt to lose their motility very quickly above or below body heat, while the saprophytic forms, unless grown for a long time at high temperature (38° C.), remain motile at higher or lower degrees. Though the former lose their motility, they are not all killed by cold. They may still be infective after freezing. Musgrave kept an encysted culture from a dysenteric stool at −12° C. for forty-five days and found it still viable at the end of that time.

A temperature of 60° C. for one hour usually kills encysted cultures of amebæ, according to Strong[1] but considerable variation has been noted in the degree of temperature necessary to destroy different strains.[2]

Enemata of quinine sulphate and saturated solution of boric acid do not affect amebæ in the intestinal canal, though a 1 to 300 solution of quinine sulphate added to the stools invariably kills them in ten minutes.

They are also destroyed in stools by weak solution of hydrogen dioxide, potassium permanganate, toluidine blue, and dilute acids.

Luttle found that 1 to 10000 hydrochloric acid and 1 to 100 silver nitrate check motility, but do not destroy parasites except after prolonged contact. Musgrave and Clegg found that in cultures treated with 1 to 2500 solution of quinine hydrochlorate the parasite quickly encysts, and in from five to eight minutes may break up and disappear; ten minutes later cultures made produced no growth of amebæ, while the bacteria grew well. Emetin has a marked effect *in vitro* and *in vivo* (see below).

**Pathogenicity.**—**Lower Animals.**—Just how pathogenic Ent. coli is for lower animals cannot be determined, as we have before stated, until a more minute study of the intestinal amebæ is made.

In regard to amebæ from tropical dysentery (presumably Ent. histolytica), they have been shown to be pathogenic for young cats, dogs, and monkeys. The infection may take place in two ways: (1) By feeding material containing the cysts; (2) by rectal inoculations of the vegetative forms. The best work done on dogs is by Harris,[1] in 1901, who found that puppies were particularly susceptible after rectal injections of fresh material from human dysentery cases. Morphine was administered before the injection in order to retard peristalsis. The disease developed in two or three days and lasted from four to sixteen days.

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[1] Osler's Mod. Med., Philadelphia, 1907.

[2] An air-dried agar plate culture of "Ameba coli" given us by Dr. Calkins who obtained if from the Philippines was viable after three years at room temperature kept in the dark. Some of our own subcultures from this plate were alive after six years in brain medium in tubes sealed with paraffin and kept at room temperature.

The chief symptoms were a bloody diarrhea and progressive emaciation. The lesions observed in the intestines on postmortem examinations were a swollen and congested mucosa, over which were scattered numerous small ulcers. In two cases there were liver abscesses.

Microscopally the mucosa first showed slight exudative and productive inflammation, followed by necrosis and desquamation of the epithelial cells and their basement membrane. At the same time the interglandular tissues beneath became swollen and small hemorrhages occurred. Great numbers of macrophages collected. Ulceration proceeded from above downward. Many amebæ were first seen in and between the epithelial cells, then in the connective tissue at the base or sides of the ulcers. Necrotic and suppurative processes producing varying degrees of suppurative inflammation may complicate the lesions.

The abscesses which form in the liver contain degenerated liver cells, polynuclear leukocytes, red blood cells, and groups of small amebæ.

As controls Harris tried rectal injections of various bacteria, including the Shiga bacillus. All gave negative results, however, and he considered that the amebæ showed their specific action very plainly. Though he did not describe the morphology of the organism from his cases with enough minuteness to identify it with Schaudinn's histolytica, he gave enough points to make the inference strong that it is the same species. Whether Entameba coli would produce similar dysentery in young dogs is yet to be proved. As stated above, Schaudinn claimed that he could produce the typical disease by feeding young cats with cysts of Ent. histolytica, but could not get the same results by feeding the vegetative forms.

Musgrave and Clegg injected "pure mixed cultures" of material from cases of clinical amebic dysentery as well as similar cultures of amebæ from various sources into monkeys and produced dysentery. Musgrave fed monkeys with encysted amebæ in bacterial cultures and obtained, in a small percentage of the cases, dysenteric stools and ulcerations in which amebæ were found without their accompanying bacteria. Kartulis, Kruse and Pasquale, and Strong injected into the rectum the contents of liver abscesses containing apparently only the amebæ and produced typical dysentery, with lesions similar to those seen in man.

Strong states that the lower monkeys and the orang-outang in the Philippines contract the disease naturally.

**In Man.**—According to Craig, about 50 per cent. of human beings harbor harmless amebæ in their intestines. Schaudinn states that he found this form of ameba in one-half the cases examined in East Prussia, one-fifth of those in Berlin, and 256 times in 385 examinations in Austria.

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[1] Am. Jour. Med. Sc., 1905.

Walker and Sellards[1] carried on an extensive study of the pathogenicity of the different forms in human beings—60 Philippine prisoners—which they divided into three groups:

1. Twenty men fed with cultures of amebæ isolated from stools or Manila water. The same ameba was recovered in cultures from several of the cases. No lasting parasitism occurred, neither did any case of dysentery result.
2. Twenty men were fed stools containing Ent. coli. No cultures were recovered; no cases of dysentery occurred, but 17 cases were parasitized.
3. Twenty men were fed stools containing Ent. histolytica. No cultures were recovered; 4 cases of dysentery occurred, and 17 cases were parasitized.

They conclude from these studies that they have furnished the proof of the pathogenicity of one form (Ent. histolytica), the non-pathogenic but parasitic nature of the other form (Ent. coli), and the saprophytic nature of cultural forms.

The disease produced by pathogenic amebæ in man is known as amebic dysentery (amebic colitis, amebic enteritis, amebiasis entamebiasis).

**Incidence.**—The disease occurs endemically in tropical countries. It is particularly prevalent in Egypt, India, and the Philippine Islands. It occurs frequently in parts of South America and southern United States. In northern United States few cases are reported, though Patterson,[2] who in 1909 described three cases (without a description of the amebæ present), and who calls attention to fifteen other cases reported as endemic in New York City since 1893, states that this disease is probably more widespread than is generally thought, and that if it were searched for more carefully more cases would be recognized. Patterson adds to his report a bibliography of cases reported as originating in North America. Sporadic cases are found in Russia, Germany, Austria, Italy, and Greece. An occasional small epidemic may occur in the milder climates. Where it is endemic the largest number of cases occur after the heavy rains have begun in early summer. Males are more frequently attacked, because more exposed to infection. It may occur at all ages, but young adults seem most susceptible. The foreign white race seems to be more susceptible than natives. Unhygienic surroundings are generally a predisposing factor, but in the Philippines individuals of all classes are likely to be attacked who do not take continuous and extraordinary precautions in regard to their drinking water. In France since the war many cases of amebic dysentery have been reported and the question of its control is an important one.

**Symptoms.**—The symptoms may be mild or severe. The disease usually runs an irregular course marked by periods of intermission and exacerbation. It may begin acutely with slight fever, griping, tenesmus and frequent stools. Occasionally, however, the outset is gradual, lasting from a few days to several weeks. The disease is generally chronic, extending over a period of a few weeks or of many years. In the mild form which is usual in children, the general conditions may be remarkably good, the only symptoms worth mentioning being the increased number of stools—two to six in twenty-four hours—which contain few to many amebæ. In the severe forms there is a loss of appetite, great emaciation, some fever, acceleration of the pulse, sweating, abdominal pains, and a decided increase of the number of stools—twenty to sixty daily. The stools are more fluid and slimy and may be bloody. They contain amebæ in varying numbers. In very severe forms the stools are watery, filled with blood, mucus, and sometimes sloughs. They vary in numbers from twenty to fifty in twenty-four hours and may contain many amebæ.

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[1] Philippine Jour. Sc., Series B., 1913, viii, 253.

[2] Am. Jour. Med. Sc., 1909, cxxxviii, 198.

The milder forms may change suddenly to the severest, and the severest may suddenly become better and completely recover.

**Tissue Changes.**—The lesions are chiefly in the large intestines. The walls are thickened in chronic cases, especially the submucosa. There are raised hemispheric areas of hemorrhagic catarrh and of ulceration. The whole of the large intestines may be affected or only more or less circumscribed areas. The amebæ pass between the epithelial cells, generally through small erosions, and they finally reach the submucosa by the lymph channels. Here reproduction takes place and the irritation to the tissue causes edema and infiltration of small spheroidal cells. This produces small elevations into the lumen of the intestines. The epithelium over these raised areas is finally eroded and then bacteria and intestinal contents help form the succeeding ulcers. The erosions or ulcerations have congested undermined margins, and yellowish-red bases. They vary in size from 2 mm. to about 2 cm. They are round, oval, or irregular in outline. The ulceration usually extends only to the submucosa, but may expose the peritoneum, and large sloughs may be cast off into the lumen of the intestines. Generally the slow inflammatory process in the submucosa leads to great thickening of the intestinal wall.

The processes may be modified in various ways by the action of other microorganisms, especially the bacteria in the feces. Healing takes place by the formation of connective tissue in the floors and by a gradual covering over with epithelium. In extensive lesions scars may form.

Peritonitis may occur with the production of an opaque gelatinous fibrinous fluid in which the amebæ may be found.

Abscesses may form in the liver (about 20 per cent. of all cases), less often in the lungs, and only occasionally in the brain and spleen. Amebæ may reach the liver through lymph channels, portal vein, and peritoneal cavity. The other organs are only slightly changed. Amebic abscesses may occur in the liver or brain with no signs of dysentery, present or past.[1]

**Source of Amebæ.**—Strong states that in Manila the greatest source of infection from amebæ is the water supply, that amebæ were cultivated from the water in large numbers in 1902, but no attempt was made to demonstrate their pathogenicity. In 1904, however, Musgrave produced dysentery in a monkey with a culture of a water ameba, though in a few experiments he was unable to infect cats from the amebæ obtained from this monkey. Practically, it is proved that people in Manila avoid being infected with amebæ if they do not drink local water, unless sterilized. Fresh vegetables as well as certain fruits may be sources of infection. Of course if pathogenic amebæ are strict parasites, the source would be only those substances contaminated with the host's excretions. A number of healthy carriers have been reported among returned troops.[2]

As dilute acids quickly kill the motile amebæ, it is probable that many of those ingested in this form are destroyed in the stomach.

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[1] Ravaut and Charpin: Presse Méd., 1919, xxvii, 65.

[2] Baylis. Lancet, 1919, i, 54.

**Immunity** to the disease may exist. It is supposed that the amebæ as they die produce toxic substances which call forth antibodies, but this has not yet been determined. The necrosis produced in the liver abscesses when bacteria are absent is an indication of the production of necrogenic substances.

**Prognosis.**—The percentage of deaths in the severe cases is quite large, especially if accompanied by abscess of the liver. Probably 25 per cent. of all cases are fatal. When treatment is begun early the prognosis is better.

**Treatment.**—Emetin has been found to be practically a specific curative treatment when amebæ are in the vegetative stage, but it does not affect cysts. In the form of ipecacuanha it is highly recommended by Manson, Dock,[1] and others, especially since the introduction of salol-coated pills which allow the remedy to reach the intestines before it is absorbed, so that large doses may be given, without inducing marked nausea and vomiting. Besides rest and diet, high enema of bisulphate of quinine have been recommended. Harris has gotten good results from hydrogen dioxide enemata diluted from 4 to 8 times with water. About a quart is injected twice daily for a week, then the amount is gradually decreased.

**Points in Diagnosis of Amebæ Found in Man.**—Examination of stools should be made as quickly as possible after they have been passed and they should be free from urine. The amebæ should be seen motile because, after encystment or death, it is often difficult to distinguish them from other intestinal contents. Bloody mucus or small pieces of necrotic tissue should be examined first, as they often contain large numbers of amebæ.

If the movements are solid a dose of salts should be given and the fluid part of the resulting stools examined.

Craig differentiates living pathogenic forms from non-pathogenic varieties by the former's (1) larger size, (2) greenish color, (3) distinct hyaline, refractive ectoplasm, (4) faint nucleus, (5) many vacuoles and red blood cells, (6) marked motility.

An absolute diagnosis of liver abscesses can often only be made by an exploratory puncture and the finding of the amebæ. If this is done the surgeon should be at hand to operate if necessary.

Ent. buccalis is usually found in the thick group of leukocytes and microörganisms collected between the teeth. The amebæ are distinguished from the leukocytes and cell detritus by (1) their large size, (2) their light, highly refractive greenish appearance, (3) their glistening red color in contrast to the yellowish red of the leukocytes when hanging drops are stained with enough of a concentrated solution of neutral red to make them appear pink.

**Differential Diagnosis between Amebic and Bacillary Dysentery.**—In amebic dysentery (1) the disease is generally chronic; (2) dysentery bacilli are usually not found in feces; (3) no severe toxic symptoms present; (4) abscess of liver frequent sequela; (5) lesion is in cecum and descending colon, not in small intestines.

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[1] Jour. Am. Med. Assn., 1902, iv, 15.

In bacillary dysentery, the finding of the bacilli, and a positive agglutination test, together with the clinical symptoms of intoxication make a positive diagnosis.

## AMEBÆ IN OTHER DISEASES.

Baelz found a very large ameba in the bloody urine and in the vagina of a twenty-three-year-old Japanese who was suffering from tuberculosis of the lung. Jurgens, Kartulis, and Posner also reported finding similar amebæ in cases of cystitis and bloody urine.

In the last few years many studies have been made on the amebæ present in pyorrhea alveolaris. The claim of Smith and Barrett,[1] Bass[2] and others that a specific ameba is the cause of this condition has not been substantiated. In examining many school children we[3] found that similar amebæ in large numbers are found in the majority of cases, but no definite relation could be traced between them and the beginning of pyorrhea. That there are several varieties of amebæ in this condition, other than Entameba buccalis is probable.

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[1] Jour. Am. Med. Assn., 1914, lxiii, 1746.

[2] Ibid., 1915, lxiv, 553.

[3] Reprint No. 37, Dept. of Health, New York City, 1915.

# CHAPTER XLII.

# SPOROZOA. CILIATA.

THE forms in the group sporozoa, which are parasitic in man, or which are of some medical interest, are, Coccidia, Nosema, Sarcocystis, Babesia, and Plasmodium malariæ and its allies. Babesia and the malarial organisms will be considered in separate chapters.

Only one genus among the ciliates is pathogenic for man.

The general characteristics have already been described in Part I.

## EIMERIA STIEDÆ (COCCIDIUM CUNICULI).

The Coccidium cuniculi is a sporozoan parasite of the rabbit, first described by Lindemann in 1865. Young rabbits are especially susceptible, and extensive epidemics may occur in breeding houses.

**Materials and Methods for Study.**—Rabbits infected with Coccidium cuniculi are often found, and the whole course of the infection may be followed with more or less ease.

A certain amount of development may be watched in hanging drops of salt solution emulsions. Sections and smears are prepared as described in Chapter III.

The cysts are stained with difficulty. It is recommended that a thin solution of Delafield's or Grenacher's hematoxylin be used for twenty-four hours followed by eosin. Heidenhain's iron-hematoxylin stain (p. 86) followed by Bordeaux red is especially good for sections.

The *symptoms* of the disease are fever, diarrhea, yellowish mucus discharge from the nose and mouth, and progressive wasting. The liver is much enlarged and shows throughout its substance variously sized grayish-white tubercles, generally surrounded by a capsule, and containing a slimy mass of degenerated host cells, in which the parasites are imbedded. The parasites are a so found in the feces and in the epithelial cells of the intestines, gall ducts, and liver. The acute stage of the disease lasts about three weeks. The contents of the coccidial tumors in animals that have withstood the infection may later be emptied, leaving only a mass of cicatricial tissue. In such animals the oöcysts may remain for a long time in the gall-bladder and intestines, and by passing out gradually with the feces may provide a source of infection for other animals. The infection is carried by food soiled with cyst-containing feces. The cysts pass with the food into the stomach, where the cyst wall and the spore sac are destroyed and the sporozoites are set free. The motile sporozoites pass through the ductus choledochus into the liver, some probably passing into the intestines and infecting the cells directly, a later infection of the intestines occurring from forms developed in the liver. The organism develops within the epithelial cells of the liver and gall ducts until the cells are finally broken down and tissue cysts are formed, within which, after more or less complicated changes, cysts of the parasite are again formed. The life cycle of Eimeria Schubergi, a similar coccidium from the intestines of a myriapod, is shown on Plate IV, Fig. III, A, 1-16.

A few cases of human infection of the liver with the Coccidium cuniculi have been reported. The Coccidium hominis Rivolta, found a few times in the human intestines, as well as similar coccidia, found in the intestines of lower animals, may belong to the same species. Haughwout[1] has described 34 cases of human coccidiosis in Manila recently.

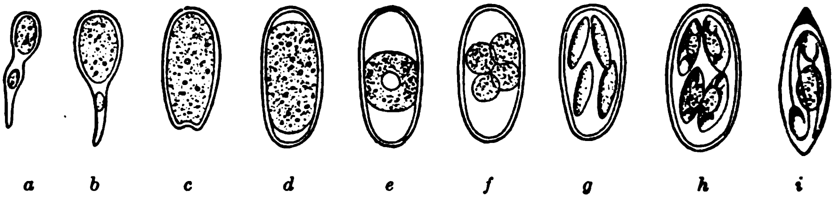

FIG. 186.—Showing spore formation in Coccidium cuniculi from the liver of the rabbit: *a* and *b*, young stage in the epithelial cells of the gall ducts (the small oval is the cell nucleus); *c*, *d*, and *e*, the fertilized oöcyst; in *d* the protoplasm is beginning to shrink away from the cyst wall, and in *e* it has contracted into a spherical form; *f*, segmentation into four sporoblasts; *g*, elongation of the sporoblasts to form spores; *h*, four complete spores in the oöcyst; *i*, single spore more highly magnified, showing the two sporozoites and a small quantity of residual protoplasm. The life cycle has been fully worked out by Simon. (After Balbiani, from Doflein.)

Coccidium bigeminum (Stiles) is found in the feces of dogs, cats, polecats, and possibly human beings. The organism is characterized by the division of the oöcyst into two united cysts, containing four spores. The size is 8$\mu$ to 15$\mu$. The life cycle is not well known.

Rhinosporidium kinealyi is the name given by Minchin and Fantham (1905) to a probable sporozoan found in the nasal mucous membrane of certain cases from India that were troubled with hemorrhagic nasal polyps. Nais reported four similar cases and Beattie another in 1906.

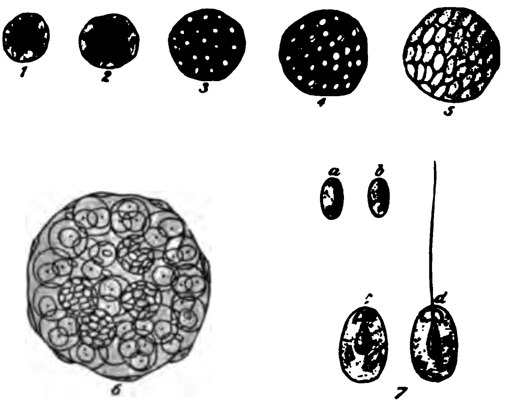

FIG. 187.—Nosema bombycis: *1* to *5*, spore formation; *6*, infected follicle of testicle; 7, spores; *a*, *b*, fresh; *c*, *d*, treated with nitric acid. The acid causes them to swell up and increase in size by at least a half, at the same time making the polar capsule distinct. In *d* the filament is extruded. (After Balbiani.)

## MYXOSPORIDIA.

The Myxosporidia belong to one of the most populous and abundant groups of the sporozoa. They show great structural variation as well as divergence in mode of life, nevertheless the members have, as a group, the following well-marked characteristics: The trophozoite is ameboid; spore formation begins at an early period and proceeds continuously during the growth of the trophozoite; the spores are produced endogenously—*i. e.*, within the protoplasm of the trophozoite, and each spore always possesses one or more very distinctive structures, "the polar capsules" (Fig. 187, *c*, *d*).

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[1] Phil. Jour. of Sc., 1919, Sec. B., xiii, 79.

The myxosporidia are habitants of fishes, reptiles, arthropods, and some other classes of animals. They infest especially arthropods, causing often most virulent epidemics. The most interesting member of this group is Nosema bombycis, the cause of silkworm disease (Pébrine). The organism forms many small spores each with one polar capsule. The spores, which are carried by the food into the intestinal canal of the caterpillar, pass through the walls of the intestines, and infect all organs. Spores found in the ovary may be carried over to the newly hatched silkworms, thus causing a further dissemination of the disease.

As far as is known man is not infected by this group.

The other member of this group, of interest here, is Nosema lophii Doflein. Its interest lies in the fact that it has been found to infect only the ganglion cells of the sea-devil, thus apparently resembling in its parasitic nature the organism causing hydrophobia.

## SARCOSPORIDIA.

This order is very little known but, considering the fact that through eating uncooked infected meat it may be found in man, though rarely, its chief characteristics should be noted here.

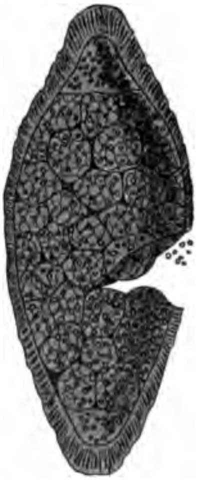

FIG. 188.—Sarcocystis tenella from the striped muscle of a swine. A full-grown cyst, showing radiately striped membrane, which is broken on the right side. (Bertram.)

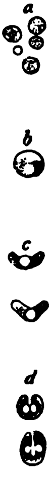

FIG. 189. — Sarcocystis miescheri, *a*, small cells from a cell group; *b*, loosening of the protoplasm from the cell wall; *c*, *d*, sickle-shaped bodies (sporozoites) formed from the small cells. (From Wasielewski.) (Mans.)

The Sarcosporidia are parasites of the striped muscles or connective tissue of some of the warm-blooded vertebrates (various birds and mammals). They are found in the adult state in elongated sacs known as "Rainey's" or "Miescher's tubes" (Fig. 188).

The trophozoite is a motionless elongated body, limited by a cuticle growing into a complicated structure. Spore-formation begins at an early stage and proceeds during the growth of the trophozoite (Neosporidia) which may become very large. The spores, which are many, are minute sickle-shaped or spindle-shaped mononucleate bodies with a delicate envelope and at one pole an oval striated body which represents the polar capsule found in the myxosporidia (Fig. 189).

In some cases the cyst wall calcifies and the contents of the cyst degenerate, with apparently no harm to the host: in other cases the cysts burst and their contents spread into the surrounding tissue, producing abscesses and tumors as with many myxosporidia, and sometimes causing the death of the host.

The symptoms of sarcosporidiosis in the pig are paralysis of the hind extremities, a skin eruption, and general systemic symptoms, such as increased temperature and pulse.

In sheep especially the disease often causes fatal epidemics. In the mouse Sarcocystis muris is a deadly parasite. Theobald Smith[1] showed that gray and white mice may become infected with Sar. muris by eating infected mouse flesh containing motile sporozoites.

Laveran and Mesnil[2] claim to have extracted a toxin (Sarcocystin) by means of glycerin or salt solution, which they have found extremely toxic for experimental animals (0.0001 gm. kills 1 kgm. of rabbit). The dried and powdered extracts are also virulent. These extracts will remain virulent for a long time in the ice-box, but will not withstand heating above 60° for any time.

Darling[3] (1909) describes a case of human sarcosporidiosis occurring in Panama from which he studied the organism and came to the conclusion that it was probably a different species from the one already described as occurring in man. He gives a good historical review. Later he decides that morphologically his human sarcosporidia are identical with Sarcocystis muris.

## CILIATA.

**Balantidium Coli** (Malmsten, 1857). The body of this infusorium is egg-shaped with a funnel-shaped mouth opening. The surface of the body is covered with a pellicle, under which is a distinct ectoplasmatic sheath containing rows of basal granules from which short, fine cilia arise.

The cloudy entoplasm contains fat and starch granules and may contain many red blood cells and other food particles from the host. Two contractile vacuoles have been seen. Posteriorly there is a small prominence marking the place where excreta are expelled. The chromatic macronucleus is bean-shaped, and the vesicular micronucleus is nearly spherical (Plate IV, Fig. iv, A–D).

Division is transverse, the macronucleus dividing by simple constriction and the micronucleus by mitosis. Conjugation has been observed. Spherical cysts surrounded by a thick membrane are formed.

*Balantidium coli* has been found in the large intestines of human beings and of swine—probably two distinct varieties. The variety occurring in human beings has been found in about 154 cases, principally in Sweden, but also in Russia, Scandinavia, Finland, China, Italy, Germany, the Philippines, and the United States. Most of these cases were suffering from severe chronic intestinal catarrh, often accompanied by bloody diarrhea. A number of observers (Strong, Brooks, De Bellard,[4] and others) think the balantidium the primary cause of the catarrh with a mortality of 23 per cent., while others believe it to be a harmless inhabitant of the intestines, or at least only a secondary excitant (Opie, Malmsten, Doflein, and others).

Schaudinn has described two additional species of balantidium found in the human intestines, which he has called, respectively, Balantidium minutum and Nyctotherus faba, probably both non-pathogenic.

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[1] Jour. Exp. Med., 1901, vi, 1; Jour. Med. Res., 1905, xiii, 429.

[2] Compt. rend. Soc. de biol., 1899.

[3] Arch. Int. Med., 1909; Jour. Exp. Med., 1910, xii, 19.

[4] Gaceta Med. d. Caracas, 1919, xxvi, 27.

# CHAPTER XLIII.

## THE MALARIAL ORGANISMS. BABESIA.

**Introduction.**—The malarial organisms are a group of protozoan parasites found to be the cause of a definite group of specific infectious fevers in man, called by the somewhat misleading term malaria, a term which signifies "bad air."

They are classed as sporozoa, order hemosporidia, and are considered by the majority of observers as forming one genus, plasmodium or hemameba.

So far as is known the only natural means by which the malarial organisms are transmitted to man is mosquitoes, genus Anopheles. A part of the life cycle of the organisms is carried on in these mosquitoes. Experimentally the disease has been produced by direct inoculation of infected human blood into humans. Accidentally it has been conveyed in transfused blood. The parasites develop in man within the red blood corpuscles which they finally destroy, thus producing the anemia and pigment granules peculiar to malarial fevers.

**Historical Note.**—The fevers caused by these organisms were recognized and studied as early as 400 B. C., but it was not until 1880 that the true nature of the dancing pigment, which had been observed long before, was determined. At that time Laveran announced that he had discovered a parasite in the blood which he claimed was the cause of the disease and he published a good description of several of the stages in the life of the organism. The public remained at first almost entirely unconvinced of the parasitic nature of these bodies. Many still believed that the bacillus described shortly before by Klebs was the cause of the fevers. Among others, Marchiafava[1] and Celli in Italy believed that Laveran's organisms represented areas of degeneration within the red blood cells, though Laveran himself demonstrated the organisms to them. When they began, however, to study the fresh tissue themselves they changed their opinion and later they published a number of valuable contributions on this subject. They gave the organism described by them the inappropriate name, Plasmodium malariæ. Laveran's researches were later confirmed by many other observers.

In 1885 Golgi showed that quartan fever depends upon a specific form of the parasite, and that the malarial paroxysm always coincides with the sporulation or segmentation of a group of parasites. Thus in a single infection with the quartan variety a paroxysm occurs every fourth day, with a triple infection on successive days, segmentation with its accompanying paroxysms occurs daily. Golgi and others soon showed that tertian fever and estivo-autumnal fevers were each due to a distinct variety of the hemameba. These varieties are at present regarded by some as distinct genera, by others as species belonging to a single genus. Councilman first called attention to the diagnostic value of the different forms which appear in the blood.

Though it had been thought for nearly two thousand years that malaria is transmitted by insects, the question was not definitely settled until Ross, in 1896, clearly demonstrated that the hematozoa of birds were transmitted by a certain species of mosquito. These investigations of Ross were soon confirmed by Grassi, Bignami, and others. Manson proved (on his son) that mosquitoes carried the infection to man. MacCallum's observations on the sexual form of halteridium were a great advance, and Bignami, Grassi, and others soon proved that all varieties of malarial fevers are transmitted from man to man by mosquitoes of the genus Anopheles. Grassi worked out the complete life cycle of the pernicious type (estivo-autumnal), while Schaudinn (1901) did the same for the tertian form. In the later classifications the genus name Plasmodium has been changed to Hæmameba.

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[1] Twentieth Century Practice, New York, 1900.

**Materials and Methods for Study.**—If a case of malaria is at hand the organism may be examined alive under the microscope by allowing a cover-glass to drop gently upon a drop of fresh blood placed upon a clean glass slide. For finer differential points, however, smears should be made (see p. 73), which may be stained by any one of several methods, such as that of Jenner, Giemsa, or Wright (see pp. 82 and 84 for details of staining). Bass and Johns[1] state that by centrifuging at high speed defibrinated or extracted blood containing malarial organisms, the protozoa rise to the top of the cell column and by this means a mass of crescents or other forms can be obtained.

**The Parasite.**—Three distinct species of malarial organisms in man have been described: Hemameba vivax (causing tertian fever), Hemameba malariæ (causing quartan fever), and Hemameba falciparum (Laverania malariæ, Hemomenas precox, causing estivo-autumnal fever). The last species has been divided by certain authors into two varieties, a quotidian and a tertian, but it is the present opinion that only one species is definitely known. See table of the chief differences between these forms (also see Plate VII).

[1] Jour. Exp. Med., 1912, xvi, 567; Am. Jour. Trop. Dis., 1915, iii, 298.

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## EXPLANATION OF PLATE VII.

Partly schematic. Drawn and rearranged by Williams, partly from Muir and Ritchie, partly from Kolle and Hetsch and partly original. Giemsa's stain.

The asexual forms show cycle of the organism in the red blood cells of the human host. They show schematically the time of fever and the day of segmentation.

*Tertian type.*

FIG. 1.—Segmented organism.

FIG. 2.—Young ring form in cell and a young form on surface.

FIG. 3.—Growing schizont; irregular form due to great motility; beginning pigment formation; red blood cell becoming paler.

FIG. 4.—Larger schizont with dividing nucleus. Red blood cells pale and stippled.

FIG. 5.—Nucleus divided into four clumps.

FIG. 6.—Further division of chromatin and formation of irregular rosette. Pigment finely granular in center.

FIG. 7.—Segmentation. Note eighteen merozoites (usually sixteen).

*Quartan type.* Shows following differences from tertian. Slightly larger, fewer segments (usually eight), and more regular. Pigment coarse. Red blood cells unaltered. Segmentation every seventy-two hours.

*Estivo-autumnal type.* Shows following differential points: Merozoites smaller and more numerous (thirty-two?); organism less motile with less pigment. Red blood cells smaller and greenish color (in fresh cells).

Sexual Forms. Show cycle of development in mosquito.

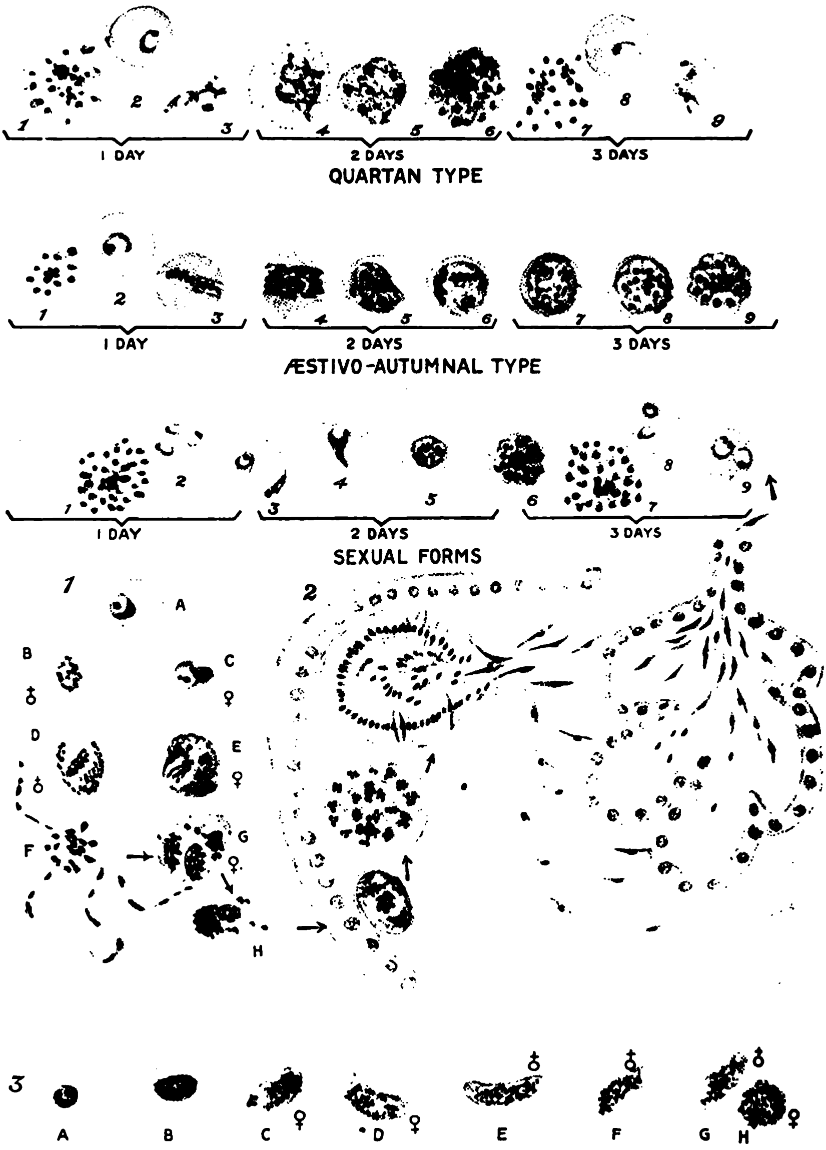
FIG. 1 (*A* to *E*).—Male (♂) and female (♀) forms of tertian type formed in human blood; *F*, flagellation of male type in stomach of mosquito; *G*, *H*, changes in female type and fertilization in stomach of mosquito.

FIG. 2—Development of sporocyst within mosquito. Liberation of sporozoites, which find their way to the salivary gland.

FIG. 3.—Sexual forms of estivo-autumnal type found in human blood, showing development of sickle-shaped bodies.

# PLATE VII

## ASEXUAL FORMS OF MALARIAL PARASITES
(SCHIZOGONY)



W. WILLIAMS DEL.

Each of these species undergoes the two phases of development already alluded to, one within the red blood cells of human beings (the asexual phase); the other within the digestive tract of the mosquito (the sexual phase). The form changes which each parasite undergoes in humans and which the benign tertian undergoes (which may be considered a type for all) in both hosts are shown on Plate VII. Briefly, they may be described as follows:

**The Asexual Cycle (Schizogony) Occurring in the Blood of Man.**— The young form is often difficult to find in fresh blood. A pale area is seen on an otherwise unaltered red corpuscle, situated usually eccentrically, about one-tenth the size of the red corpuscle or about one-fourth its diameter, when at rest presenting a rounded appearance, but usually actively ameboid, throwing out distinct pseudopodia never remaining long in the same focal plane, frequently dipping, so to speak, into the substance of the corpuscle. It is often called the hyaline form because it is free from pigment, but it is not hyaline in the proper sense of the term. It is also called the ring form, because of its resemblance to a ring in stained preparations; but it is never a true ring. The ring appearance is produced by the formation of a large food vacuole. The organism continues to grow either on or in the red blood cell.

Rowley[1] claims that the organisms never enter the red blood cells. They only feed from the surface. Her beautiful demonstrations show that possibly the majority of them do this.

The forms intermediate between this and the segmentation stage appear in the fresh blood simply as larger parasites, which are readily found on account of the reddish-brown pigment granules that they contain. These granules begin to appear several[2] hours after the organism has infected the red blood cell. At this time the organism is usually actively ameboid and the granules have a lively dancing motion, due to protoplasmic currents in the parasite. The infected corpuscle is swollen and paler. In the estivo-autumnal form the infected red blood cells are smaller than normal.

When the parasite has approached nearly to its full growth, it occupies the greater portion of the corpuscle, which is now more difficult to make out. The pigment is still more evident, so that this form is therefore most readily found. At this stage ameboid movements are not so active. When full growth is reached, segmentation occurs. The forms up to the period of segmentation are called schizonts.

The morphological changes which have been going on in the parasite preparatory to segmentation are best studied in properly stained smear preparations. In the living organism they become presently sufficiently distinct to be followed; the pigment gathers more or less centrally into a compact mass, and a peripheral notching indicates that the parasite is preparing to divide into a number of segments called merozoites; the number of these segments varies in the different species. (See table.) Suddenly the segments separate as small spheroidal

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[1] Jour. Exp. Med., 1914, xix, 450.

[2] See table for number of hours in each species.

TABLE SHOWING CHIEF DIFFERENCES BETWEEN THE SPECIES OF MALARIAL ORGANISMS (GENUS HÆMAMEBA) FOUND IN MAN.

| Name of organism. | Size of parasite up to segmentation (schizont). | Motion of young schizont in corpuscle. | Time of appearance of melanin granules and their arrangement. | Shape of segmenting parasite, number of segments, and site of segmentation. | Asexual cycle complete in | Sexual forms. | Incubation period. | Effect on human host tissues. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|
| H. vivax (parasite of tertian fever). | 1μ to slightly larger than normal red blood cell (may occasionally be almost twice size) | Markedly active | 6 hours; first scattered, then gathered in centre; finely granular; actively dancing | Irregular mulberry; 12-24 (average 16); peripheral circulation | 48 hours | Gametocytes spherical; no crescents; male ½ size of female, which is 1½ times size of red blood cell | About 14 days | Pale, granular; slightly enlarged red blood cells; finely granular pigment formed from metamorphosed hemoglobin | Double infection may cause a paroxysm every day, thus giving clinically a quotidian type of fever. |
| H. malariæ (parasite of quartan fever). | 1μ to little less than size of red blood cell | Not very active | Within a few hours; collected in zone on periphery; coarsely granular; slight dancing | Regular daisy in shape; 6 to 10 (average 8); peripheral circulation | 72 hours | Gametocytes spherical; fewer than in vivax; about size of red blood cell; no crescents | About 3 weeks | Red blood cells may be slightly shrunken | Triple infection may cause a paroxysm every day, thus giving clinically a quotidian type of fever. |
| H. ▼ falciparum (parasite of estivo-autumnal or pernicious fever). | Smaller than others, from very small to ⅔ diameter corpuscle | Active; slightly less so than tertian type | Within 24 hours; small amount, 2 or 3 coarse granules usually central; non-motile or sluggish | More or less symmetrical daisy; 10 to 32 very small; chiefly in bone marrow and viscera | 24 to 48 hours | Gametocytes crescentic, short and plump; ⅔ size of red cell | About 10 days | Red blood cells unstained, greenish, shrivelled (crenated) and darkened; stained (Giemsa) salmon color. | |

bodies, the young parasites. A corpuscular remnant and the pigment float away and are ultimately ingested by phagocytic cells. The young parasites attach themselves to red corpuscles as before and the human cycle is repeated.

In a suitably stained preparation the young parasite (see Plate VII), appears to be a disk consisting of a central pale, unstained area, known as the achromatic zone, and of a basic (blue) periphery, the body, including a metachromatically stained, rounded, compact (red) chromatin mass, the nucleus, which tends to give the parasite the form of a signet ring.

Later stages up to a certain number of hours show simply changes in size and outline of the body. The nucleus then divides by simple mitosis. Later it breaks up by amitotic division into an increasing number of small masses. By the time the chromatin division is completed the chromatin masses will have assumed a rounded form, and will be seen to exhibit ultimately the same strong affinity for certain dyes which is seen in the compact chromatin body of the young ring-like form. At this stage the heretofore scattered pigment appears in one clump. Good technic will always show a corpuscular remnant even at this time. The achromatic zone mentioned will be seen to develop with the chromatin, and when the next step, namely, the division of the body of the parasite, is seen to be completed, there will be as many achromatic bodies as there are chromatin bodies, each division having a share of the basic mother-body, each representing the young parasite (merozoite).

A certain number of the full-grown parasites do not segment and these are the forms which commence the life cycle in the mosquito. These forms grow to produce the sexual forms, the macrogametocyte, or female organism, and the microgametocyte, or male organism. When mature these forms are generally larger than the mature schizont of the same species, the female organism being usually larger than the male and containing more food granules and a smaller nucleus. In the estivo-autumnal forms they are crescentic in shape, while in the other species they are spherical. In the circulating blood of human beings they show no further changes except to become freed from the corpuscle; but when the blood containing them is withdrawn and exposed for a short time to the air, an interesting series of changes in the microgametocyte is observed. The crescentic bodies are transformed into spherical bodies; the pigment of the microgametocytes becomes actively motile, due to internal agitation of the chromatin fibrils, which presently emerge as flagella-like appendages. Their movements are very rapid, causing corpuscles to be knocked about, and finally they become detached as the microgametes, or male elements, and go in search of the female element. In the withdrawn blood of birds, one may actually observe the process of conjugation in slide preparations even without the aid of a moist chamber and heat. This transformation of male bodies never occurs in the human blood. It will be seen that it belongs to the sexual cycle which occurs in the stomach of the mosquito.

**The Sexual Cycle (Sporogony) Developing in the Mosquito.**—The common mosquito, often day-flying, belongs to the genus Culex; it cannot carry human malaria. It is easily distinguished from its night-flying or dusk-flying relatives. Anopheles (the malaria-carrying mosquitoes comprise about eight genera of the subfamily anophelinæ), by its assuming a different posture on the perpendicular wall. While the Culex holds the body more or less parallel with the surface, the body of the Anopheles stands off at a marked angle. Other differential points are the following (see Figs. 190-199):

Wings of Culex are unspotted; those of Anopheles are spotted (except in one rare species).

The proboscis of Anopheles points toward the resting surface, while that of Culex does not do so.

Anopheles species bite usually in the early evening, while those of Culex bite almost at any hour of the day.

The male mosquito is readily told from the female by its plumed antennæ, those of the female being inconspicuous.

The eggs and the larvæ of the two genera are quite distinct, as may be readily seen by glancing at Figs. 190 and 191. The anopheles mosquitoes breed in practically any kind of a collection of water, though some species prefer slow-running water to quiet pools. The best known domestic carriers are usually found in barrels and cisterns.

If an ordinary mosquito (Culex) is allowed to imbibe the blood of a malarial patient whose blood shows gametocytes there will be simply a digestion of such blood in the mosquito, and no development of the malarial organisms results. If, however, certain species of Anopheles ingest such blood, immediate changes follow. It should be remembered that only female mosquitoes are blood sucking; hence they alone can be responsible for the spreading of the disease. It should also be remembered that if the blood imbibed by the anopheles does not contain gametocytes, though it may contain earlier stages of the malarial organisms, no amount of such blood can cause general infection of the mosquito. The sexual cycle is similar in all species of the parasite.

The flagellation of the male parasite described above will promptly take place in the stomach of the anopheles, 4 to 8 microgametes being formed; these conjugate with the female element (Plate VII) in a manner comparable to the impregnation of the ovum of higher animals by spermatozoids. The macrogametocyte becomes a macrogamete by the formation of a reduction nucleus which is thrown out of the organism.

The product of conjugation, the oökinet (zygote), remains for a number of hours in the juices of the chyme stomach, changing gradually from a spherical, immobile body into an elongated motile wormlet (Plate VII, Sexual Forms, 1 II). This penetrates the epithelial lining of the stomach and rests in the tunica elasticomuscularis (Plate VII, Sexual Forms, Fig. 2); here it changes into an oval then into a round body, which grows in the course of the next few days enormously, forming a cyst which projects into the body cavity. Meanwhile the

Fig. 190

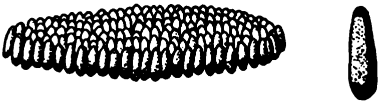


Fig. 191

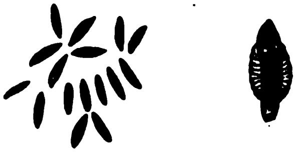


Fig. 192

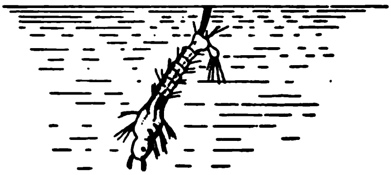


Fig. 193

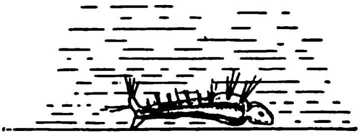


Fig. 194

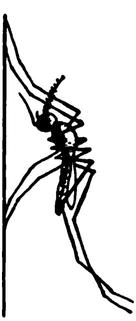


Fig. 195

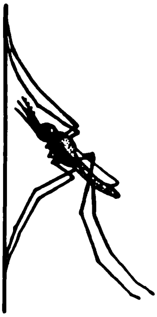


Fig. 196

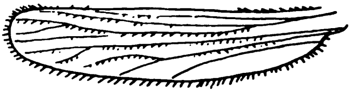


Fig. 197

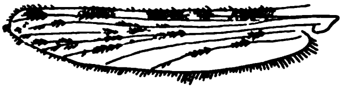


Fig. 198

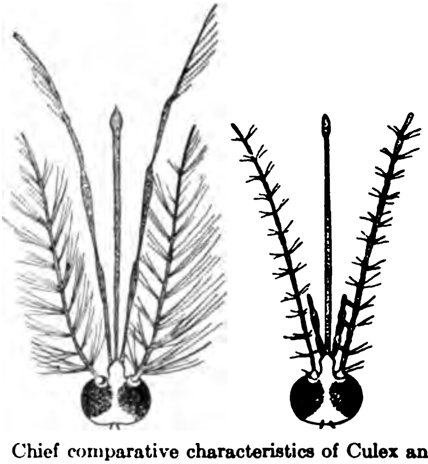
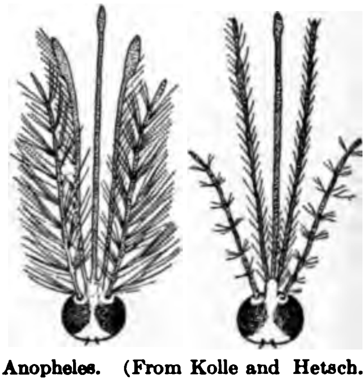


Fig. 199



Chief comparative characteristics of Culex and Anopheles. (From Kolle and Hetsch.) Egg of Culex, Fig. 190, laid together in "small boat," those of Anopheles, Fig. 191, separate and rounded. Larva of *C.*, Fig. 192, hangs nearly at right angles to water surface, those of *A.*, Fig. 193, are parallel to surface. Body of *C.*, Fig. 194 when resting is held parallel to wall in a curved position, that of *A.*, Fig. 195, stands at an angle of about 45° and is straight; wings of *C.*, Fig. 196, are generally not spotted, those of A., Fig. 197, are spotted. In *C.* the palpæ, Fig. 198, of the female are very short, of the male are longer than the proboscis; *in A., Fig. 199* the proboscis of both sexes are about of equal length.

chromatin will have become very active. It will have divided into numerous nuclei, which become arranged around inactive portions, and filamentous sporozoites develop from this chromatin and surrounding protoplasm (Plate VII, Sexual Forms, Fig. 2). These sporozoites ultimately fill the cysts, which rupture, setting them free into the cavity of the mosquito's body; they then are carried by the lymph to all parts of the body of the mosquito and thus reach a glandular structure in the thoracic cavity of the insect, the so-called salivary gland (poison gland), in which they accumulate in large numbers. This gland is in immediate connection with the biting and sucking apparatus. If, now, such an infected mosquito "bites" a human being, the lubricating fluid of the puncturing apparatus will carry sporozoites into the latter's blood and the human cycle begins. The stages of development in the mosquito require from seven to ten days, but only when the temperature is favorable.

**Cultivation.**—Bass and Johns[1] announced in 1911 that they had succeeded, by a rather complicated method, in obtaining a certain amount of development of H. vivax in the test-tube (see special media for details of method). Essentially, defibrinated malarial blood is used, to which is added ½ per cent. dextrose. Bass and Johns state that the layer of serum about the sedimented red blood cells must not be too deep (½ inch) and that the leukocytes must be removed if more than one generation of the parasite is wished. The Thompsons[2] (1913) state that they have gotten several generations by less attention to these details.

They draw the malarial blood into the sterile test-tube (10 c.c.) in which there is a thick wire, and 0.1 c.c. of a 50 per cent. aqueous solution of glucose. The blood is defibrinated by gently stirring the wire for five minutes. The wire and clot are removed, and the blood is poured into smaller tubes, 1 inch in each. Rubber caps are placed over cotton plugs. These tubes are kept at 37° to 44° C. The corpuscles settle slowly, leaving about ½ inch clear serum. They have found it unnecessary to remove the leukocytes by centrifuging. They state that the malarial organisms are not destroyed by the leukocytes in the tube but by the changes in the serum. Their organisms grow through the whole depth of the layer of red blood cells.

**Effect on Man (Pathogenesis).**—As the organism grows at the expense of the red blood cells the principal change is in the blood. Melanemia, or the formation of pigment granules from the destroyed red blood cells, is one of the most characteristic features of malaria. As the disease progresses the red corpuscles show varying changes in form and hemoglobin content, not only the infected corpuscles, but others as well, thus showing that the organism produces either primarily or secondarily some toxic substances. The pigment occurs in two forms, melanin and hemosiderin. The second only gives the reaction for iron and is found in the internal organs, while the first is found everywhere in the circulating blood. The pigment is taken up by the leukocytes. There is usually a definite reduction of both red and white blood corpuscles, which is more marked in tertian and quartan malaria than in estivo-autumnal. There is a relative increase in the number of mononuclear leukocytes. The spleen shows marked hyperplastic inflammation and pigmentation. In intense estivo-autumnal cases the capillaries of the brain and other organs may be filled with the parasites. We have observed parasites also in the large nerve cells of the brain.

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[1] Jour. Exp. Med., 1912, xvi, 567; Am. Jour. Trop. Med., 1915, iii, 298.

[2] Ann. Par. and Trop. Med., 1913, vii, 509.

**Toxin Production.**—The relationship between segmentation and paroxysm is always noted in tertian cases, and it is reasonable to suppose that the occurrence of the paroxysm is referable entirely to the liberation of toxic substances resulting from metabolic activity of the parasite within the corpuscle. That there should be a toxic product seems highly probable, and its amount must be considered in heavy infections. Cases showing an infection of 1 to 5 per cent. of all corpuscles are not infrequent; the destruction of from 50,000 to 200,000 or more corpuscles per cubic millimeter of blood leads to the rapid deglobularization of the blood; hence the deficiency in numbers; add to this the effects of the metabolic products, and little is left to the imagination to explain the pronounced anemia.

**Immunity** from malaria appears to exist as natural and acquired immunity.

**Prophylaxis.**—The fact that, with the extermination of the malaria-carrying mosquitoes, malarial fevers in man would be made impossible, remains established; the parasite must have its chance of rejuvenescence in the mosquito's stomach.

The various methods of extermination are fully described in books which go minutely into the subject. The method of giving small doses of quinine to human beings exposed to *Anopheles,* and of thus getting rid of the organism itself within man, of disinfecting man, should be considered. In hot climates especially, where it is practically impossible totally to destroy the breeding places of the mosquitoes by drainage or oiling, this method is especially serviceable. Bass[1] has reported recent extensive efforts in this direction. In these countries, too, the use of adequate screening is of marked value.

**Points of Diagnosis.**—By a study of the parasite taken from the circulating blood the examiner should be able to tell not only the species present, but also the progress the disease is making. Malarial parasites can always readily be found in recent primary infections, and it is usually only in old cases that the search becomes difficult; one is, however, generally rewarded by finding them if one looks long enough for them.

A helpful sign is the finding of pigment in mononuclear leukocytes, which are seen about the time of a chill or of the period symptomatically corresponding to it. Free pigment cannot be used as a means of diagnosis, as it may be impossible to tell it from dirt or dust. In a primary infection of *long standing* the gametocytes may be found, and in relapses and in those cases treated by quinine, many atypical forms appear. A small dose of quinine may drive all parasites except the sexual forms out of the peripheral circulation; at all events the finding of them becomes, in the absence of gametocytes, a matter of time and experience, especially also as they may be much altered in appearance. The part most and first affected is the blue-staining body; later follow eccentricities of the chromatin, such as multiple bodies; and dwarfing, just such changes as might have occurred in time, if the body had been allowed to combat the parasite without the aid of drugs. In both cases the fever curve becomes atypical. It should be remembered that there is no quotidian form originating in this country. Quotidian paroxysms occurring here are either a double tertian, or a triple quartan infection. The notion that the parasites can be found only at the time of the paroxysm is still in the minds of many; it is erroneous. The gametocytes are quite resistant to quinine and other drugs, and it appears as if cases in which these forms are seen are much more prone to relapse than promptly treated recent primary infections. The macrogametocytes may remain quiescent for years in the blood, and then under certain conditions, probably through parthenogenesis, may again begin to develop and multiply, thus bringing about relapses.

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[1] Jour. Am. Med. Assn., 1919, lxxiii, 21.

In the estivo-autumnal forms the crescentic gametocytes are generally few, but at times large numbers of them develop. Of course they are absolutely characteristic. The young parasites are more or less characteristic in stained preparations (Plate VII). There may be as many as seven parasites in one corpuscle. Later the few heavy pigment granules are characteristic.

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## DESCRIPTION OF PLATE VIII.

(After Goldhorn.)

1. Typical young tertian form; the corpuscle shows incipient degeneration; corpuscle to left above shows a blood platelet.

2. Abnormal young form, showing small accessory chromatin body.

3. Two parasites; one normal young form; the second a large form in crenated corpuscle is an unusual abnormal form with very large achromatic area.

4, 5, 6. Estivo-autumnal parasites; single, double, and triple infection; central elongated chromatin bodies. These forms are about the largest usually seen in the peripheral blood; no degeneration of corpuscle.

7. Tertian parasite, about ten hours old; marked degeneration of corpuscle.

8. Double infection of a corpuscle in tertian fever; marked degeneration of corpuscle.

9, 10, 11. Large tertian parasites showing division of chromatin previous to segmentation.

12 and 14. Complete segmentation of tertian parasite.

13. Double infection of corpuscle, one parasite reaching maturity, but showing unusually small segments; the second one atrophied.

15. Tertian parasite, old case; while the parasite is only half-grown, the chromatin has split into several compact masses. Degeneration of infected corpuscle.

16. Dwarfed tertian parasite, smaller than a red corpuscle, but showing five compact chromatin bodies; resemblance to quartan rosette.

17. Microgametocyte of tertian malaria; prominence of blackish pigment surrounding a large acromatic zone in which the microgametes lie coiled up.

18. Tertian macrogametocyte.

19 to 23. Crescentic bodies of estivo-autumnal malaria.

19. Typical gametocyte; pigment surrounding achromatic area; no chromatin shown; the "bib" is present. (Male?)

20. Semiovoid gametocyte. (Female?)

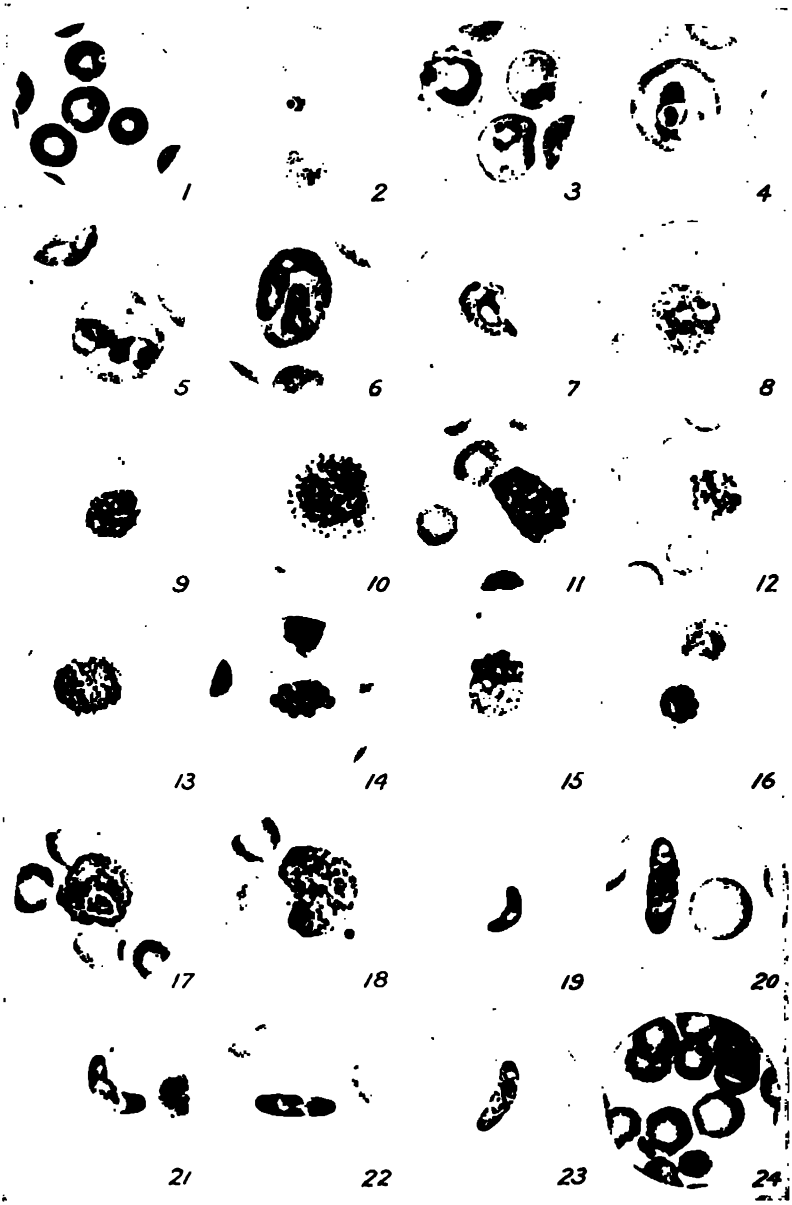
21. Pigment removed. Elliptical achromatic area in which the microgametes are seen.

22 and 23. Pigment removed; chromatin more compact; possibly female elements.

24. From a case of pernicious malaria with rich infection; only hyaline forms in peripheral blood. Below, a large blood platelet.

NOTE.—As the amplification is not uniform, a comparison of the parasites with the blood corpuscles shown should be made in order to have a correct conception of their size.

PLATE VIII



Photographs of Tertian and Estivo-autumnal Malarial Parasites in Different Stages of Development.
(Goldhorn.)

In fatal cases the formation of crescents may not take place; the blood infection with young parasites is then enormous, every field of the microscope showing numbers of them.

In the study of estivo-autumnal fever, as well as in that of the other forms, it is to be remembered that crescents when found indicate that the disease is of some standing, for such sexual forms are not formed until the asexual propagation is waning. The recognition of these ovoidal and crescentic bodies is easy. But as there are no readily discoverable pigmented forms in the peripheral blood in the early stages, it is necessary to be thoroughly familiar with the young estivo-autumnal forms. Polychrome staining for them cannot be too much recommended, as there is little that is characteristic about them when they have been stained with methylene blue alone. Many a serious error has been made by adhering to the antiquated idea that parasites should be looked for in the fresh blood, as these young, non-pigmented, so-called hyaline forms cannot be readily recognized by the inexperienced, while it is an easy matter to know and classify them when properly stained.

The recognition of the quartan parasite in its early stages in the fresh blood is not as difficult as that of the tertian form, because the outline is more distinct; but in stained preparations it is often indistinguishable from the latter. The living ameboid young form or schizont is more refractive than the young living tertian schizont, more like the estivo-autumnal form, and it is just as sluggish in its movements. Then, too, the corpuscle is often shrunken and looks as if it contained more hemoglobin than in the case of infection with the tertian parasite.

The growing parasite rapidly becomes pigmented, but it shows fewer, larger, less motile pigment granules than the corresponding tertian one; moreover, the pigment is arranged around the periphery of the organism, while in the tertian form it is distributed throughout the protoplasm. The quartan parasite is apt to form a band across the infected corpuscle. Segments are few in number, as a rule, and the parasite remains dwarfed while the infected red blood cells are normal in size. The segments are generally arranged symmetrically around the central pigment, giving the so-called daisy or marguerite appearance to the parasite at this stage (Plate VII).

In tertian fever the granular degeneration which the infected corpuscles early undergo is diagnostic. In the first few hours it resembles the ordinary granular stroma degeneration with basic affinity, while it is later seen that the affinity of the then more numerous granules is more acid, or, at least, the staining is no longer orthochromatic, the blue being superimposed by a red; in other words, these granules stain later metachromatically. The greater the loss or transformation of the hemoglobin the greater the number of granules. This holds good only for tertian parasites, the estivo-autumnal variety causing practically no appreciable change, though the same technic is used.

**Malarial-like Parasites in Other Animals.**—Two genera of protozoa closely related to the malarial organisms have been found in birds:

(1) the proteosoma or hemoproteus; (2) the halteridium; both found in owls (*Hemoproteus noctuæ* Celli and Sanfelice). Points in their life history have been brought out by various observers, especially by Ross and by MacCallum. The complete life cycle of both forms, as worked out by Schaudinn, is considered by him and his followers to be of fundamental importance to the understanding of the relationship of blood parasites. Schaudinn states that these organisms pass through a flagellate stage in the intestinal tract of the common mosquito (*Culex pipiens*) which has previously fed on owls infected with the intracellular organisms (halteridium and hemoproteus). Novy considers that this mosquito flagellate stage of Schaudinn is simply a growth of trypanosomes in the mosquito's intestinal tract which are normally found there, and that Schaudinn did not sufficiently control his work to warrant his conclusions.

Malarial-like organisms have been found also in monkeys, cattle, dogs, and frogs, but they have been little studied.

**Blackwater Fever.**—This is a condition which occurs frequently, especially in Europeans in tropical countries. Its etiology has been the subject of much discussion. The chief symptoms are fever, hemoglobinuria, delirium, and collapse. It frequently ends in coma and death. A few consider it a disease entity, but the majority of observers are inclined to believe it the terminal stage of a severe malarial infection. It is frequently associated with the demonstration of the malarial organism, but they are not always found. It may be that the invasion of the ganglion cells of the brain by the malarial organisms are the chief cause of the symptoms, aided in certain cases by the lysemia noted by Christophers and Bentley.[1]

## GENUS BABESIA (PIROPLASMA).

It was not until 1888 that there was a hint as to the real nature of the actual cause of "Texas fever" (bovine malaria, tick fever, hemoglobinuria) and allied diseases which attack field cattle in many parts of the world. Then Babes described inclusions in red blood cells in Roumanian cattle sick with the disease, though he did not decide upon the nature of the organism. No new studies were reported until 1893, when Theobald Smith and Kilborne[2] gave such a complete description of this disease and its cause as occurring in Texas cattle that little concerning it has since been discovered.

These authors describe as the cause of Texas fever, pigment-free ameboid parasites appearing in various forms within the red blood cells of infected animals. The organisms may be irregularly round and lie singly or they may be in pear-shaped twos, united by a fine line of protoplasm.

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[1] Jour. Trop. Med., 1907, x, 323.

[2] U. S. Dept. of Agri., 1893, Bull. No. 1.

Because of these double pear-shaped forms Smith and Kilborne named the organism Pyrosoma bigeminum[1] and they placed it provisionally among the hemosporidia. These authors also showed that the contagion was carried by a tick (see p. 604). Their work has been corroborated by many investigators in different parts of the world. Hartmann places this genus in his new order Binucleata, and he considers it an important form for showing the relationship of the endocellular blood parasites to the flagellates. Schaudinn, in 1904, was the first to call attention to the occurrence of nuclear dimorphism in B. canis and bovis, and Luhe, Nuttall and Graham-Smith,[2] Breinl and Hindle, and others have confirmed this observation. The second nuclear mass is generally in the form of a small granule similar to the blepharoplast of undoubted flagellates.

**Morphology of the Parasite.**—In the examination under 1000 diameters of fresh blood of sick cattle, according to Smith and Kilborne, are seen, in the red blood cells, double pear-shaped forms and single rounded or more or less irregular forms. The size varies, though generally it is the same among the bodies in the same red blood cell. The average size is $2\mu$ to $4\mu$ long and $1\frac{1}{2}\mu$ to $2\mu$ wide. The pointed ends of the double form are in apposition and generally touch, though in unstained specimens a connection between them cannot be seen. The axis forms either a straight line or an angle. The protoplasm has a pale, non-granular appearance, and is sharply separated from the protoplasm of the including red blood cell. The small forms are generally fully homogeneous, whereas the larger ones often contain in the rounded ends a large rounded body, $0.1\mu$ to $0.2\mu$ in size, which is very glistening and takes a darker stain. Within the largest forms in the center of the thick end is a large round or oval body, $0.5\mu$ to $1\mu$, which sometimes shows ameboid motions. Piana and Galli-Valerio (1895 and 1896) and other observers have since described definite ameboid motion of the whole parasite. The motion of the whole parasite on the warm stage is not produced by the formation of distinct pseudopodia, but by a constant change of the boundary. The changes can succeed each other so quickly that it is scarcely possible to follow them with the eye. The motion may persist for hours. The single ones show motion, while the double ones remain unchanged.

The parasites take most basic anilin stains well. The Romanowsky method or its modifications give the best results (Plate IV, Fig. III, B). Stained by this method the smallest forms appear as tiny rings, about one-sixth the diameter of the red blood cell. A part of the rim takes the red nuclear stain, the rest is blue. In the large mature pear-shaped organisms a loose mass of chromatin is at the rounded end and a dense, compact mass is situated nearer the pointed end. These mature, pear-shaped forms, Nuttall states, are the mark of distinction between *Piroplasma* (Babesia) and other intracorpuscular blood parasites. These pyriform bodies are generally present in pairs, and occasionally, in the acute form of the disease, sixteen pairs may be seen in a single blood cell.

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[1] The generic name Pyrosoma, already in use for a well-known Ascidian genus, was altered to Piroplasma by Patton in 1895. In the meantime Starcovici (1893) had given the name Babesia bovis to the form described by Babes; and as this form seems to be identical with that described by Smith and Kilborne the correct name of the genus should be Babesia while the species parasitic in cattle should be called Babesia bigemina.

[2] Jour. Hygiene, 1905, v, 485; 1906, vi, 586; 1917, vii, 232; also Parasitology, 1909, ii, 215, 229, 236.

The number of red cells infected is about 1 per cent. of the whole. If the number increases to 5 per cent. or 10 per cent., it generally means the death of the animal. The parasites quickly disappear from the blood after the disappearance of the fever. In fatal cases many parasites are found in the red blood cells of the internal organs. They vary in number according to the stage at which death occurs, are most abundant in the kidneys (50 to 80 per cent. of all red corpuscles infected), and are found in fewer numbers in the liver, spleen, and other internal organs.

R. Koch[1] has described a bacillar form which he found in large numbers in red blood cells of acute fatal cases in East Africa. Between these and the pear-shaped forms he found all grades. This variety is probably a distinct species.

Flagella-like appendages in Babesia have been described by several observers as occurring in the blood in mammals. More frequently they have been seen in the tick and in attempted cultures. They have been interpreted by some (Hartmann, Calkins) as possible microgametes, by others (Breinl and Hindle) as true flagella, and by others (most observers) as fine pseudopodia.

**Transmission.**—Smith and Kilborne showed that the infection is caused by a species of tick, *Margaropus annulatus*, Say (*Boöphilus bovis*) (Fig. 200), and Kossel gives *Ixodes redivius* as the tick causing transmission of the germ in the hemoglobinuria of Finland cattle.

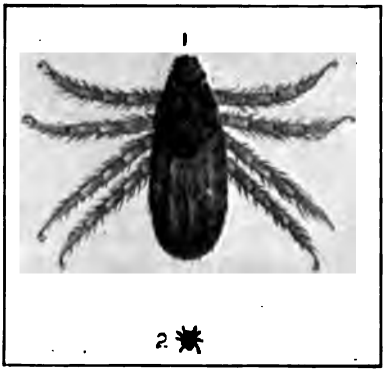

Fig. 200.—No. *1*, Texas fever tick, Margaropus annulatus (Boöphilus bovis). × 15.4. No. *2*, natural size. (Mohler.)

The ticks feeding upon the blood of cattle and other mammals become sexually mature at their last moult. They then pair, and the fertilized females, after gorging themselves with the blood of their host, drop to the ground. Each female then lays about 2000 eggs, and within the shell of each egg a large quantity of blood is deposited to serve as food for the developing embryo. The female then shrivels up, becoming a lifeless skin. The newly hatched larvæ containing in their abdomens some of the mother-blood, crawl about until they either die from starvation or have the opportunity of passing to the skin of a fresh host. If the mother-tick has drawn its supply of blood from cattle infected with piroplasma, her larvæ are born infected with the parasite and become the means of disseminating the disease further. This mode of dissemination explains the long incubation period of the disease (forty-five to sixty days—thirty days—for the development of the larvæ and the remainder for the development of the parasite within the host). It is possible that the tick embryo acquires the infection secondarily from the blood it absorbs in the egg, and that the parasites do not pass through the ovum itself as in *Nosema bombycis*. This species of tick *M. annulatus* has been found also on sheep and ponies.

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[1] Ztschr. f. Hygiene, 1901, xlv, 2.

So far it has not been possible experimentally to inoculate animals other than cattle with these parasites. Calves withstand the infection better than older animals and a certain degree of immunity is reached in some of the older cattle in infected districts. The piroplasmata taken in by such animals may remain as harmless parasites for some time. If, however, such cattle are weakened from any cause, their resistance to the organism may be lowered and they may therefore pass through a more or less severe attack of the disease.

Nuttall and Graham-Smith report a study of canine piroplasmosis, and have drawn a cycle showing the usual mode of multiplication in the circulating blood. They consider B. canis a species distinct from B. bovis and B. pitheci (found by Ross, in 1905, in blood of a species of cercopithecus) though no morphological differences are given.

Christophers has described probable sexual stages of development in the tick R. sanguineus, so that he has drawn a complete life cycle of the organism.

**Symptoms of the Disease.**—Fever (40° to 42° C.), anorexia, weakness, increased pulse and respiration, decreased secretion of milk, hemoglobinuria at the height of the fever, causing the urine to appear dark red like port wine or darker. The urine may contain albumin even if the hemoglobinuria is absent, but there are no red blood cells present, the color being due to the coloring matter of the blood only. There is icterus of the mucous membranes if much blood is destroyed.

**The prognosis** varies in different epidemics from 20 to 60 per cent. Death may occur in three to five days after first symptoms appear. Recovery is indicated by a gradual fall of the fever.

**Treatment.**—Quinine in large doses seems to have helped in some epidemics. Nuttall, Graham-Smith, and Hadwen have reported curative effects from trypanblau in both canine and bovine babesiosis (Piroplasmosis).

**Prophylaxis.**—Stalled cattle are not infected, but it is impracticable to keep large herds of cattle stalled. If the cattle are kept from infected fields for one or two years and other animals (horses and mules) are allowed to feed there the ticks may disappear. The burning of the field for one season may have a good effect. If animals cannot be taken from infected fields such fields should be enclosed.

Ticks on animals may be killed by allowing the cattle to pass through an oil bath (paraffin, cottonseed oil, etc.), whereupon the ticks die from suffocation. The bath should be repeated after a week in order to kill any larvæ which may have developed. All animals sent from infected regions should receive this treatment. Animals apparently healthy before the treatment, after the disturbing influence of the bath often develop the disease in an acute form and die.

Certain birds in Australia seem to feed on the ticks, therefore such birds might be propagated.

Various attempts have been made to give protection by the inoculation of fresh (not older than two or three days) blood from slightly infected animals. Some partial results have been reported, especially when the inoculations were made during the cold months. In Australia the inoculation of defibrinated blood from animals which have just recovered from the infection, but whose blood still contains some parasites, has been tried. So far no absolute protection has been produced, neither does the parasite-free serum of animals which have entirely recovered from the disease seem to contain protective qualities.

**Cultivation.**—Thompson and Fantham[1] have reported successful development in the test-tube after the method of Bass and Johns for malaria.

**Other Blood Organisms.**—Blood organisms similar to those described in the hemoglobinuria of cattle have been found in cases of red water fever of cattle in England. They also occur in monkeys, dogs, sheep, horses, and pigeons. Nocard and Motas, who have made an extensive study of these parasites in the malignant jaundice (hemoglobinuria, malaria, or biliary fever) of dogs, state that though the parasites are morphologically similar to those infecting cattle, yet it is impossible to infect cattle or any other animal tried with them. They must therefore be considered a physiological variety.

Strong[2] and his collaborators reported that in an extensive study of *oroya fever*, a tropical disease, they had determined the cause to be a Babesia-like organism which they called *Bartonia bacilliformis*.

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[1] Ann. Par. and Trop. Med., 1913, vii, 621.

[2] Jour. Am. Med. Assn., 1913, lxi, 1713; 1915, lxiv, 805 and 965.

## CHAPTER XLIV.

## SMALLPOX (VARIOLA) AND ALLIED DISEASES.

**Introduction.**—The diseases smallpox, cow-pox, vaccinia, horse-pox, sheep-pox, if not identical, are closely allied. Indeed, the following facts seem to prove that at least cow-pox and variola are very closely related, if not essentially the same disease: *First,* smallpox virus inoculated into calves produces, after passage through several animals, an affection exactly similar to cow-pox. The successful inoculation of the first series of cattle from smallpox is a matter of great difficulty, but so many experimenters have asserted that this has been done that there seems to be no doubt as to its truth. In our laboratory not one of many attempts to accomplish it has been successful. *Second,* both when occurring in nature and when produced by experiment the lesions of the two diseases are similar. *Third,* monkeys have been successfully protected against either disease by previous inoculation of the other; also, observations go to show that human beings inoculated with cow-pox vaccine are not susceptible to inoculation with smallpox virus, and that those who have within a varied time passed through an attack of smallpox cannot be inoculated successfully with cow-pox vaccine. These facts seem positively to prove that the two diseases are produced by organisms originally identical, one being modified by its transmission through cattle, the other through human beings.

Variola is perhaps the most regularly characteristic of the diseases of man. It is highly infectious and is controlled only by vaccination. Notwithstanding the fact that we know definitely the exact site of the infective agent in this disease and that certain experimental animals are susceptible to inoculation of the material containing the infective agent, most investigators are still undecided in regard to the nature of the chief exciting factor. A few, however, claim that certain bodies found chiefly in the epithelial cells of the skin and mucous membranes in the specific lesions are protozoa causing the disease.

**Definition.**—Smallpox (Synonyms: Variola, la variola, Blattern, Pocken, Vajuola) is an acute infectious disease characterized by an epidermic eruption of vesicles and pustules which, upon healing, produce cicatrices of varying extent and depth.

**Historical Note.**—The first undoubted description of the disease was given by Rhazes in the tenth century, but it is evident that he did not consider it a new disease. To trace its original home seems to be impossible. It may have developed first in certain regions in Asia and Central Africa where it is at present endemic and is said to be uncontrolled by vaccination. Many outbreaks of the disease in the United States can be traced directly to the importation of African negroes.

The disease, carried by the intercommunication, principally of war and commerce, was widespread when Edward Jenner showed conclusively in 1798 that vaccination with cow-pox afforded protection. Now the few cases of variola that occur are seen in those who, through neglect or ignorance (sometimes wilful), have not been vaccinated.

**Etiology of Variola and Cow-pox.**—It has been repeatedly shown that no bacteria similar to any of the known forms have a causal relation to these diseases. In our own laboratory we are able, by the inoculating of rabbits' skins, to produce extremely active vaccine virus in large quantities, absolutely free from microörganisms which grow under the conditions of our present methods of bacterial cultivation. Such pure active vaccine, when emulsified in equal parts of glycerin and water and filtered through two or three thicknesses of the finest filter paper, gives a slightly opalescent filtrate, which in the hanging drop under high magnification shows many very tiny granules with an occasional large one, and in smears shows no formed elements giving characteristic stains. This filtrate, from which no growth was obtained on artificial culture media, when rubbed over a freshly shaved rabbit's skin after the method of Calmette and Guérin, or when used to vaccinate human beings, gives an abundant typical reaction.

These facts show that some, at least, of the infective forms cannot as yet be made to grow outside of the body, that such forms are very minute, and that they do not stain characteristically with our usual methods of staining. In a few experiments we were unable to filter the virus through a Berkefeld filter under forty pounds' pressure, but this may have been due to the fact that we did not dilute our virus sufficiently. Since then Bertarelli and a few others have reported that the virus is slightly filtrable under pressure. Park and Wilson, however, on later experiments (1913) still obtained only negative results.

Steinhardt, Israeli, and Lambert[1] (1913) report that evidence of multiplication of germs may be obtained by growing the virus on living tissue *in vitro*.

Since Guarniéri, in 1892, claimed that certain inclusions present in the epithelial cells of the lesions of smallpox in a rabbit's cornea (Fig. 201) were parasites, much attention has been given to the study of these bodies, commonly known as "vaccine bodies," yet opinions still differ as to their nature. Among the more important studies of these bodies are those, on the one hand, by Councilman[2] and his associates who believe them to be protozoa, and, on the other, by Ewing,[3] who believes that all of the forms so far described are degeneration products, some specific, others not.

Calkins, working with Councilman, thinks that his original tentative cycle is too elaborate. He still firmly believes that the bodies are protozoa, but that they belong among the rhizopoda and not among the microsporidia where he first placed them.

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[1] Jour. Inf. Dis., 1913, xiii, 294; 1914, xiv, 87.

[2] Jour. Med. Res., 1904, xii, 1; Osler's Modern Med., Philadelphia, 1907, ii.

[3] Ibid., 1904, xii, 509.

Prowazek and others believe that the organisms of this group of diseases, as well as of rabies, scarlet fever, trachoma, and a few others, are all minute coccus-like forms which have the power of producing an envelope from the host cell substance, such envelope with its contained organism constituting the specific body which others have called a protozoön. Prowazek[1] calls the group Chlamydozoa and says they probably stand between the bacteria and the protozoa in systematic classification. From our studies on this whole group of diseases we have come to the conclusion that there is no close relationship between the trachoma bodies and the intracellular bodies of rabies, smallpox and scarlet fever (see pp. 461 and 620).

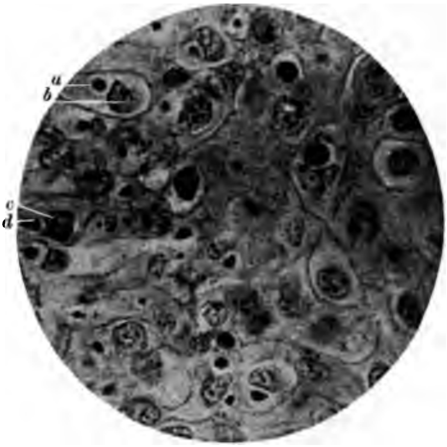

FIG. 201.—Epithelial cells of a rabbit's cornea, containing many "vaccine bodies." Tissue fixed three days after inoculation with smallpox virus. *a* and *d*, vaccine bodies; *b* and *c*, nuclei. × 1500 diameters.

In our own work on sections, which has extended irregularly over a period of several years, we have gotten results which are somewhat confusing, principally because of the non-uniformity of the appearances of these bodies, both by different methods of demonstration and by the same methods at different times. There is no doubt that, whatever the nature of the bodies, they are easily affected by methods used for fixing, hardening, and staining them. This accounts in part for the varied results reported. However, in the most perfectly prepared specimens, judged according to the appearance of the red blood cells, leukocytes, and tissue cells at a distance from the lesions, we have found that the vaccine bodies, especially in corneal infection, show a more or less constant series of changes, somewhat similar to those described by Calkins in his "gemmule formation" and by Tyzzer in his development of the vaccine bodies.

Our best results on corneas have been obtained with the following technic: Fix in Zenker's fluid for from four to eight hours; wash in running water overnight; place in 95 per cent. alcohol (changing in two hours to fresh) for twenty-four hours, then in absolute alcohol for twenty-four hours. Imbed in paraffin. The cuts should be from $3\mu$ to $5\mu$ thick. Stain with (1) eosin and methylene blue (Mallory)—eosin half an hour, methylene blue two minutes; (2) Heidenhain's iron hematoxylin; or (3) Borrel modified by Calkins.

The vaccine bodies may be studied for a short time in the living cornea by rapidly excising an inoculated cornea, spreading it on a shallow agar plate and dropping a thin cover-glass over it. The structured bodies are very clearly differentiated from the rest of the cell contents, and interesting changes have been observed in them. Too little work has been done, however, by this method, to draw any further conclusions in regard to their nature. Councilman and Tyzzer photographed these living cornea bodies with the ultraviolet light, and the structure came out just as the chromatin structures of known living cells do.

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[1] Deutsch. med. Wchnschr., 1907, xxxiii, 1285.

**Pathogenesis.**—FOR LOWER ANIMALS.—Various animals seem to contract the disease, or a modification of it, in nature. Horse-pox, sheep-pox, and cow-pox, all show similar pathological changes. Experimentally, probably all mammals are susceptible, though in varying degrees. Most of them are more sensitive to vaccinia than to variola. The epidermis of rabbits, for instance, shows a beautifully typical eruption after inoculation with vaccine virus, while material from smallpox eruptions produces only diffused redness. The corneal "take," however, in both instances, is similar in intensity. Monkeys are equally susceptible to both forms of the disease.

FOR MAN.—Without vaccination human beings seem to be equally susceptible to infection with variola, whatever their race or their condition in life or in whatever part of the world they live.

**Immunity.**—The immunity caused by successful vaccination is not permanent, and varies in its duration in different individuals. Although it usually gives protection for several years and may give it for ten or fifteen years, it is not well to count on immunity for more than one year and whenever one is liable to exposure it is well to be vaccinated. If this vaccination is unnecessary it will not be successful, while if it is successful we have reason to believe the individual was open at least to a mild smallpox infection.

**Protective Substances Present in the Serum of Animals after Successful Vaccination.**—It has been frequently shown that the blood serum of a calf some days after an extensive vaccination possesses feeble protective properties, so that the injection of one or two liters of it into a susceptible calf prevents a successful vaccination. A further and more convincing fact has been demonstrated by Huddleston and others, namely, that when active vaccine is mixed in certain proportions with serum from an animal which had just recovered from a successful vaccination, and the mixture is inoculated into a susceptible animal, there is no reaction.

**The Preparation of Vaccine.**—The following is the method employed at the New York City Health Department.

**Seed Virus.**—This may be prepared by any one of the methods given below:

1. *Glycerinized bovine virus* which has been ripened for two or three months. Such virus becomes attentuated after a varying number of calf-passages.

2. *Rabbit Virus.*—Rabbits vaccinated with stock bovine virus. This may also result in deterioration.

3. *Human Virus.*—This consists of the serum collected on sterilized bone slips from the vaccine vesicles of previously unvaccinated children. This produces good vaccine on calves; it is manifestly difficult to secure.

4. *Glycerinized vaccine* from calves which have been vaccinated with an emulsion of human vaccine crust. This also gives good virus, but it necessitates the constant collection of human crusts.

5. *Human-Calf-Rabbit Seed.*—This is the most economic, efficient, and reliable seed yet found by us. It is produced as follows: Crusts are collected from healthy children about nineteen days after successful vaccination. These crusts are cut up and emulsified with boiled water to a mucilaginous paste. This humanized seed is inoculated into an area about 6 inches square upon the abdomen of a calf, the remainder of the calf being vaccinated in the ordinary way. The pulp from this special area is separately collected and glycerinized in the usual way. It is then tested bacteriologically and clinically. This bovine virus from human seed is now used in a dilution of 1 part to 12½ parts of normal salt solution to vaccinate rabbits. The seed is rubbed thoroughly on the freshly shaven skin of the back. Five days after vaccination the pulp is removed with a curette, weighed and emulsified in a mortar with the following solution: glycerin 50 per cent., sterile water 49.5 per cent., and carbolic acid 0.5 per cent., in the proportion of 1 part of pulp to 8 parts of the solution. Four rabbits should yield from 15 to 20 c.c. of this emulsion, an amount sufficient to vaccinate one calf. The regular or commercial supply of vaccine is produced by vaccinating calves with this rabbit seed in the manner to be described.

**Animals.**—The preferable animals are female calves, from two to four months of age, in good condition and free from any skin disease. These can easily be vaccinated on the posterior abdomen and insides of the thighs by placing them on an appropriate table. It is possible that, on account of the character of the available supply, older animals may be desirable, but the calves take more typically and are more easily handled. When an animal is too old to be thrown and held without difficulty it may be vaccinated on the rump each side of the spine; but the skin there is tougher than on the posterior abdomen and inside of the thighs, and the resulting virus, though efficient, is not so easily emulsified.

**Vaccination.**—The hair should be clipped from the entire body when the animal is first brought into the stable and the calf should be cleaned thoroughly, including the feet and the tail. Just before vaccination the posterior abdomen and insides of the thighs are shaved and the skin beneath washed in succession with soap and water, sterilized water and alcohol, and then dried with a sterile towel. On this area there are now made superficial linear incisions with a sharp knife, about a fourth of an inch apart. After they have been made they should be dried with a sterile towel or with sterile cotton and rubbed with the seed virus by means of a sterile spatula. About 20 c.c. of seed is used for each calf.

**Care of the Calves.**—The calves are inspected by a Department veterinarian at the time of purchase and during the period of detention previous to vaccination. After the vaccine has been collected the calves are immediately killed and their organs examined by the veterinarian. If, at autopsy, an animal be found tuberculous or otherwise diseased, the vaccine is discarded, The vaccine stable at Otisville, N. Y., is a new building with screened windows and concrete floors and stalls, which are kept flushed with water to wash away the dejecta of the animals. The calves stand upon raised racks of galvanized iron. They are fed on milk, no hay or straw being used for any purpose. The calves are vaccinated and the vaccine pulp collected under careful aseptic precautions in a separate operating room, which has tiled walls and a concrete floor. The vaccine pulp when collected is placed in sterile glass containers, sealed, packed in ice and shipped at once by express to the vaccine laboratory.

**Collection.**—On the fifth or sixth day, depending upon the rate of development of the vaccine vesicles, they should be ready for collection. The entire shaved area is washed with sterile water and sterile cotton, and the crusts are picked off. The soft, pulpy mass remaining is then curetted off with an ordinary steel curette and the pulp placed in a sterilized vessel. The pulp should be mixed with four times its weight of glycerin and water (50 per cent. glycerin, 49 per cent. water, 1 per cent. carbolic acid). This is done by placing the pulp in a mortar and adding the solution gradually. The mixture of vaccine pulp and diluent is pressed through a 40-mesh sieve with the pestle several times until there is no residue on the sieve. Then it is passed twice through a 100-mesh sieve. The emulsion so produced is stored in 100 c.c. bottles at 2° to 4° below zero Fahrenheit. After the necessary bacteriological tests for purity (see below) it can be put up for issue in vials. Capillary tubes require special means of filling, and small vials filled and corked answer the purpose admirably.

**Testing of Finished Product.**—The virus as it comes from the calf may contain the hay bacillus, skin staphylococci, possibly streptococci and perhaps B. Welchii (Bacillus capsulatus aërogenes). The presence of tetanus spores is always feared but they are rarely found. After storage of the virus at the low temperatures mentioned above, the action of the carbolic, glycerine and the cold has usually freed the virus from bacteria. Before it is released for the clinical test it is subjected to the following bacteriological tests and must be free from contamination.

*Purity Tests.*—(1) Plating on agar and counting of the colonies after incubation at 37° C. for two days, then reincubating at 20° C. (room temperature) and recounting the colonies.

2. Inoculation of 2 per cent. glucose broth in fermentation tubes. The presence of growth and gas are noted. Stained smears show whether or not streptococci are present, also whether any of the organisms resemble B. Welchii. If so, they are tested for aërobic growth on ordinary agar. Should no growth appear the rabbit test (p. 610) is made.

3. Test for tetanus bacillus—Meat infusion broth in fermentation tubes is planted with a portion of the ground-up pulp to which no carbolic has been added with the glycerine solution. The tubes are incubated at 37° C. for nine days. Any tube showing growth is used for an inoculation test in either mice or guinea-pigs. One c.c. of the nine-day culture is injected subcutaneously and the animal watched daily for six days for symptoms of tetanus.

4. Test for streptococcus—A guinea-pig is inoculated subcutaneously with the freshly prepared virus and observed for ten days for evidences of streptococcus infection.

A more rapid freeing of the vaccine virus from bacterial contamination may be accomplished by the use of brilliant green dye in the glycerine carbolic solution in a concentration of 1 to 10,000. This has no appreciable effect on the potency of the virus. Its color, however, renders it unsuitable for commercial purposes. It would be most useful in an epidemic when large amounts of vaccine are needed in a short time, or for experimental work.

**Clinical Tests for Potency.**—After all the laboratory tests for purity have been made and found satisfactory, *and not until then,* the vaccine is ready for use upon human beings, provided it be found to be efficient. To determine this point, fifteen inoculations of the vaccine are made upon previously unvaccinated children. These must all show a perfect take in order to pass the vaccine as up to standard. A clinical test of such vaccine is made every two weeks thereafter so long as the vaccine is on the market. If one of these tests fails before the end of the period of guarantee, the vaccine is called in.

**Keeping of Vaccine.**—Bulk vaccine is kept in cold storage at a temperature of 4° to 8° F. below zero.

Vaccine which has been put up in packages ready for issue is kept in an ice-box at a temperature of 33° to 40° F.

# CHAPTER XLV.

# RABIES. YELLOW FEVER.

## RABIES.

**Introduction.**—Rabies (synonyms: Hydrophobia, Lyssa, Hundswuth, Rage) is an acute infectious disease of mammals, caused by a specific virus, and communicated to susceptible animals by the saliva of an infected animal coming in contact with a broken surface, usually through a bite. The name rabies (Latin) is given to the disease because of its most frequent and characteristic symptom—furor or madness. Hydrophobia (Greek, fear of water) is another name commonly used, which is also given because of a frequent symptom of the disease, the apparent fear of water. Lyssa is a Greek word meaning hydrophobia. Within the gray nervous tissue of rabid animals are peculiar protozoön-like structures known as "Negri bodies" which are diagnostic of rabies. The nature of these bodies is still a question of dispute (see below).

**Historical Note.**—Rabies is probably one of the oldest diseases in existence, but because of the occurrence of so few human cases, and because the disease develops so long after the bite, its source was for a long time not known nor was it recognized as a separate disease. Hippocrates does not mention it in his writing, but Aristotle about fifty years later (about 300 B. C.) speaks of its being purely an animal disease and being carried by the bite of one animal to another. Celsus, in the first century, was the first to give in writing a detailed description of human rabies. He speaks of it being produced by the bite of rabid animals and states that the wound must be thoroughly bathed and than burned with a hot iron in order to prevent the development of the disease, for after symptoms appear death always follows. As Celsus was not a physician he probably obtained his knowledge from writings which have since been lost. Other writers soon after gave very true descriptions of the symptoms and handling of the disease.

Many hundred years passed after this without adding anything to our knowledge of the disease, though authors on the subject were numerous. Van Sweiten in 1770 observed the paralytic form of rabies in human beings. At this time several authors, among them Morgagni and Zwinger, believed that the bite of a dog which was not suffering from rabies might produce the disease in man. In 1802 Bosquillon brought forth the original idea that belief in the existence of infectious material in rabies was a chimera and that hydrophobia was simply due to fright. This false idea had adherents for a long time; even now, by a few people, it is thought to be a true one.

Among the host of good observers who studied the disease during the latter part of the nineteenth century, Pasteur stands out as the discoverer, in 1880, of the fact that the disease may be prevented by inoculating gradually increasing doses of the virus into the person or animal bitten. This treatment with some modifications, the details of which will be given later, is still used, though many efforts have been made to develop an efficient serum treatment. Pasteur, as well as numerous other investigators, tried to discover the specific cause of rabies, but all of the results were negative. The importance of making a quick diagnosis had become so evident that the efforts of many workers were directed toward this end alone.

Pasteur and his immediate followers relied for their diagnosis entirely upon rabbit inoculations, and this meant a fifteen to twenty days' wait before the patient knew whether or not the treatment he was receiving was necessary. In 1898 this time was shortened to about nine days in our laboratory by Wilson, who found that guinea-pigs came down with the disease much more quickly than rabbits. From time to time it has been thought that certain histological findings were diagnostic; for instance, the "rabic tubercles" of Babes, and the areas of "round- and oval-celled accumulation in the cerebrospinal and sympathetic ganglia" of Van Gehuchten and Nelis were said to be specific, but further study has shown that they are not absolutely specific for rabies. In many cases the whole picture of the grosser histological changes is sufficiently characteristic to warrant the diagnosis of rabies, but often it is not so.

It was not until Negri, in 1903, described certain bodies (Negri bodies) seen by him in large nerve cells in sections of the central nervous system, that anything was found which seemed absolutely specific for hydrophobia. Negri claims that these bodies are not only specific for rabies, but that they are probably animal parasites and the cause of the disease. We independently found the same bodies.

This work, especially so far as the diagnostic value of these bodies is concerned, has been corroborated by investigators in almost all parts of the scientific world, among them workers in our own laboratory who not only determined their worth in diagnosis, but investigated their nature.

In our work[1] emphasis was placed upon the fact that the demonstration of the "Negri bodies" by our "smear method" (see p. 616) wonderfully simplified the process of diagnosis. As a result of our studies we concluded that the Negri bodies are not only specific for rabies, but that they are living organisms, belonging to the protozoa, and are the cause of the disease; giving as our reasons the following facts: (*a*) They have a definite characteristic morphology; (*b*) this morphology is constantly cyclic, that is, a definite series of forms indicating growth and multiplication can be demonstrated; (*c*) structure and staining qualities, as shown especially by the smear method of examination, resemble those of certain protozoa.

Since this report was published many more cases of rabies have been more or less studied by us and our former conclusions have been more firmly established. Indeed, the evidence as to animal nature of these cell inclusions seemed so convincing that Williams, in 1906, gave them the name Neurocytes hydrophobiæ.[2] Calkins has since studied these bodies and agrees with Williams as to their nature. He called attention to the similarity between their structure and that of the rhizopoda.

A number of observers, however, still believe that the Negri body as a whole is principally the result of cell degeneration and that the specific organism may be contained within it. Prowazek includes rabies with his "chlamydozoan diseases" (see p. 465). To anyone who has made a long and minute study of the two diseases, however, there can be no question in regard to the essential difference between the "trachoma bodies" and the "Negri bodies."

**Material and Methods for Study.**—In New York one may still frequently obtain fresh brains of rabid animals, from veterinary hospitals or from the laboratories handling this material. Two methods have been used in helping to study the principal site of infection.

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[1] Jour. Inf. Dis., 1906, iii, 460.

[2] Proceedings of the New York Pathological Society, 1906, vi, 77.

(1) Animal inoculations. (2) Sections and smears.

The first method is used as a decisive test in diagnosis when results from the second method are doubtful.

The technic of the *smear method* used at present in the Research Laboratory of the New York City Health Department is as follows:

1. Glass slides and cover-glasses are washed thoroughly with soap and water, then heated in the flame to get rid of oily substances.
2. A small bit of the gray substance of brain chosen for examination is cut out with a small sharp pair of scissors and placed about 1 inch from the end of the slide, so as to leave enough room for a label. The cut in the brain should be made at right angles to its surface and a thin slice taken, avoiding the white matter as much as possible.
3. A cover-slip placed over the piece of tissue is pressed upon it until it is spread out in a moderately thin layer; then the cover-slip is moved slowly and evenly over the slide to the end opposite the label. Only slight pressure should be used in making the smear, but slightly more should be exerted on the cover-glass toward the label side of the slide, thus allowing more of the nerve tissue to be carried farther down the smear and producing more well-spread nerve cells. If any thick places are left at the edge of the smear, one or two of them may be spread out toward the side of the slide with the edge of the cover-glass.
4. For diagnosis work such a smear should be made from at least three different parts of gray matter of the central nervous system: (1) From the cortex in the region of the fissure of Rolando or in the region corresponding to it in lower animals (in the dog, the convolution around the crucial sulcus); (2) from Ammon's horn, and (3) from the gray matter of the cerebellum.
5. The smears are partially dried in air and the method of fixation and staining given on p. 86 is applied to them.

With this method the Negri bodies stain magenta, their contained granules blue, the nerve cells blue, and the red blood cells yellow (Plate IX, Fig. 1).

Other methods we have found useful for staining smears are: (1) Giemsa's (p. 83), by which generally the "bodies" are a blue and the contained granules are azur. The cytoplasm of the nerve cells stains blue also, but with a successfully made smear the cytoplasm is so spread out that the outline and structure of most of the "bodies" are seen distinctly within it. The nuclei of the nerve cells are stained red with the azur, the nucleoli a dull blue, the red blood cells a pink yellow, more pink if the decolorization is used (Plate IX, Fig. 2). The "bodies" have an appearance of depth, due to their refractive qualities. (2) eosin-methylene-blue method of Mallory (p. 86). With this method of staining, the cytoplasm of the Negri bodies is a magenta, light in the small bodies, darker in the larger; the central bodies and chromatoid granules are a very dark blue, the nerve-cell cytoplasm a light blue, the nucleus a darker blue, and the blood cells a brilliant eosin pink. With more decolorization in the alcohol the "bodies" are not such a deep magenta, and the difference in color between them and the red blood cells is not so marked.

In the technic of the *section* work (p. 86) the most important point is the time the material is allowed to remain in Zenker. According to our experience two hours' fixation is not enough, three to eight hours is very good, and with every hour after eight hours the results become less satisfactory. Left in Zenker overnight the tissue is granular and takes the eosin stain more or less deeply, both of which results interfere with the appearance of the tiniest "bodies," especially of the very delicate, minute forms found by us in sections from fixed virus.

The sections may be stained by the eosin-methylene-blue method of Mallory (p. 85). In the sections made in this way we have been able to demonstrate clearly very minute forms, as well as good structures in the larger forms Giemsa's method for sections may also be used. Mann's method, recommended by others, has not given us such good results.

Harris[1] has published a staining method for both sections and smears, which

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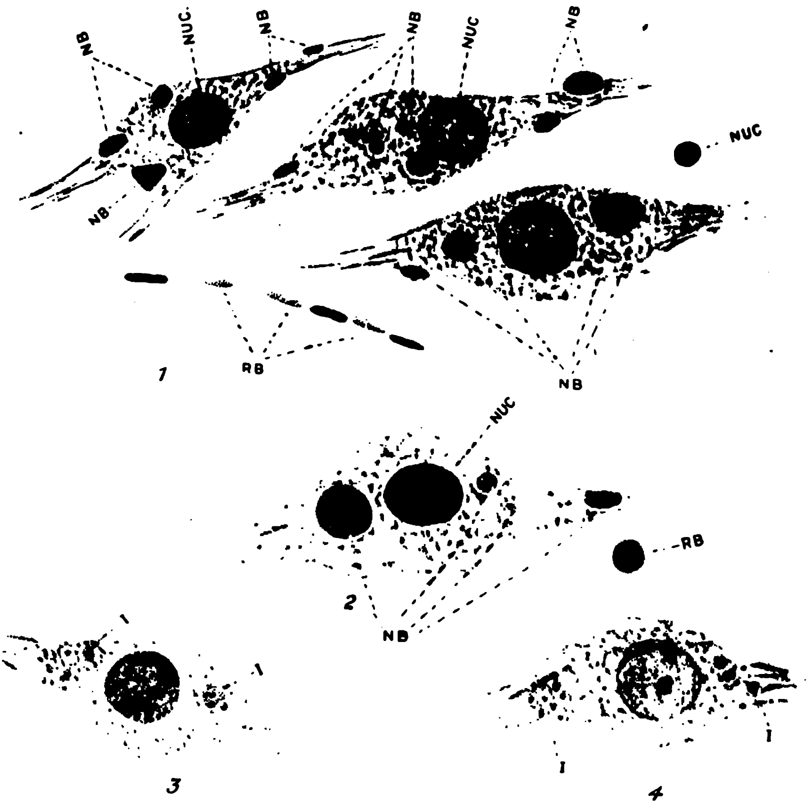
[1] Jour. Inf. Dis., 1908, v, 566.

# PLATE IX



A. W. WILLIAMS, DEL.

Nerve Cells in Spreads from Ammon's Horn.
Magnification 1200 diameters.

Figs. 1 and 2 from dog "street rabies" show "Negri bodies" (NB); Fig. 8 from non-rabic cat, and Fig. 4 from dog distemper show indefinite inclusion (I) that might be mistaken for Negri bodies by the inexperienced. Negri bodies are structured, more intensely staining, and more refractive. Figs. 1, 8, and 4 are stained by fuchsin-methylene blue mixture, which stains Negri bodies (NB) red with blue granules, nucleus (NUC) of nerve cells blue, and red-blood cells yellow (RB). Fig. 2 is stained by Giemsa's mixture, which stains Negri bodies a robin's egg blue with red granules, nucleus of nerve cells red, and red-blood cells salmon pink.

brings the larger bodies out clearly, but which does not seem to give enough differentiation between the smaller bodies and the nucleoli of the nerve cells.

**Morphology of the Negri Bodies.**—The largest forms measured are about 18$\mu$ and the smallest about 0.5$\mu$. They are round, oval, oblong, triangular, or ameboid. The latter are more numerous in the fixed virus of rabbits and guinea-pigs. Their structure is shown especially well in smears. Whatever the variety or species of animal infected, the bodies present the same general characteristic structure; *i. e.*, a hyaline-like cytoplasm with an entire margin, containing one or more chromatin bodies having a more or less complicated and regular arrangement.

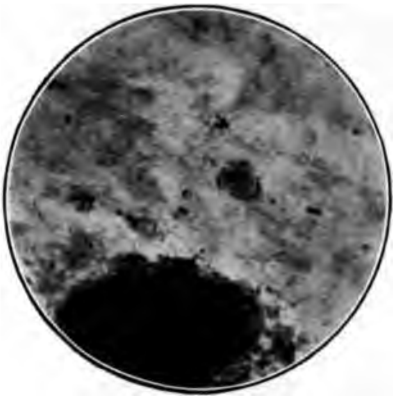


FIG. 202.—Negri body showing central chromatin with ring of small granules. × 2000.

Their structure varies to a certain extent with their size. In fixed virus, with an occasional exception, only tiny forms are found. These are rounded or sometimes wavy in outline, as if possessing slight ameboid motion, sometimes elongated, extending along the rim of the host-cell nucleus, or along one of the nerve fibrils, as if moving there; with eosin and methylene blue they take a delicate light magenta stain, very similar to that taken by the small serum globules in the bloodvessels. Many of the organisms, however, show a small chromatin granule, situated more or less eccentrically, sometimes on the very rim of the body. In the larger forms the granule is large, in the smaller it cannot always be seen; some of the larger forms show from two to several granules and occasionally there is a body with the definite central body and the small granules about it.

*Detailed Characteristics of Structure in the Large Forms* (Fig. 202).—In smears, as well as in sections, the *cytoplasm* appears quite homogeneous; there is no evidence of a reticulum or of a granular structure outside of the definite chromatoid granules. The smears, however, have brought out one important point in regard to the cytoplasm more clearly than the sections, and that is that it is more basophilic than acidophilic in staining qualities. With the Giemsa stain, as we have already seen, it takes the methylene-blue stain more than the eosin red, and even with the simple eosin-methylene-blue stain the protoplasm appears as a deep magenta unless much decolorized.

In studying the *central bodies* of these organisms, as they appear in the smears, one of the first things noticeable is that they are not surrounded by a clear space—that there is no sign of a vacuolar appearance in the body as there usually is in the sections. We notice next that in the great majority of the organisms the central body stands out clearly, as decidedly different in structure, and slightly so in staining qualities, from the chromatoid granules which surround it. The general type of the structure of the central body is that of many well-known protozoan nuclei; that is, the chromatin is arranged in a more or less granule ring around the periphery of the central body or nucleus, leaving an achromatic or more acid-staining center in which is situated, generally eccentrically a varying-sized karyosome. There are a number of variations from this principal type, according to stage of development (Plate IX, Figs. 1 and 2).

Fragmented particles seem to be leaving the nucleus in certain forms, and in this way presumably the chromatoid granules are produced.

The *chromatoid granules* are most frequently arranged in a more or less complete circle about the nucleus. They are somewhat irregular in outline and size, being occasionally ring-shaped, sometimes elongated, often in twos, due probably to active changes of growth and division. They take generally a more mixed chromatin stain than the chromatin of the nucleus.

**Evidences of Division.**—All stages in transverse division are seen. Many evidences of budding are also seen. The chromatoid granules seem to divide and pass out with part of the cytoplasm as a bud. This budding or unequal division appears to take place very early in the growth of the organism and to continue throughout growth until the parent body forms a mass of small organisms which may then break apart at the same time. The budding accounts for the number of small and large forms in a single cell (Plate IX, Fig. 1).

**Number.**—They vary in number according to the stage of the disease and to the infectivity of the part.

**Site.**—They are situated chiefly in the cytoplasm and along the fibers in the branches of the large nerve cells of the central nervous system. In parts of smears which are more broken up the bodies may appear as if lying free, and it is these bodies, if the pressure is not too great in smearing, that show the structure best. In some cases the structural bodies are distinctly localized in small scattered areas of the central nervous system. We have always found bodies in the spinal cord in abundance, but here they are especially prone to be localized in discrete groups of cells.

Manouèlian[1] has found them in the ganglion cells of the salivary glands.

That the organisms are present in various glands of the body (salivary thyroid, suprarenal capsule, etc.) is shown by the virulence of emulsions from these organs. Cows' milk (Westbrook, McDaniel) and blood (Marie) have also been shown to be occasionally virulent.

**Cultivation of the Rabies Organism.**—Many attempts have been made to produce artificial cultures, but no reports have been so far corroborated. Noguchi[2] states that he has obtained virulent cultures on the twenty-first transplant. Williams,[3] Gurley,[4] Krauss and Barbara,[5] and Volpino[1] have not been able to get the same results. The latest claimants for artificial cultures are the English workers, Bradford, Bashford and Wilson,[2] who state that they have obtained filtrable globoid bodies in pure cultures. These claims have just been withdrawn.[3]

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[1] Ann. de l'Inst. Past., 1914, xxviii, 233.

[2] Jour. Exp. Med., 1913, xviii, 314.

[3] Forscheimer's Therapeusis, 1916, 2d. ed., v, with references.

[4] Coll. Studies, New York City Health Dept., 1914, p. 15.

[5] Deut. Med. Wchnschr., 1914, xl, 1507.

**Diagnosis of Rabies.**—In our laboratory, for the past thirteen years, or since we have used the smear method in routine diagnosis, there have been many thousand cases in all examined, including suspected rabies and controls.

In all of our work controlled by careful animal inoculations we have never yet failed to have typical rabies develop in animals inoculated with material showing definitely structured Negri bodies. Negative results after inoculation with such material must be interpreted at present as due to some error in technic, such as regurgitation, or hemorrhage at the time of inoculation, emulsion improperly made, not enough of the virulent material taken because of localization of the organisms, etc.

Possibly individual resistance of the animal inoculated might play a part. We have used principally guinea-pigs, and some of them have shown enough irregularity in regard to the time in which they have come down with the disease to suggest a varied individual susceptibility, if other factors can be ruled out.

On the other hand, material in which we have failed to demonstrate typically structured bodies has produced rabies. All of this material, however, since we have improved our technic, has shown suspicious small forms similar to those found in rabbit-fixed virus. But any decomposing brain may also show in smears, bodies very similar to these tiny forms, therefore it is difficult to rule out rabies in such cases. Of course the animal test will probably always have to be used with brains that are too decomposed to show any formed elements except bacteria, unless a reliable chemical test can be discovered. Brains from animals dying of distemper may show small non-structured forms, somewhat like "fixed virus" forms (Plate IX, Figs. 3 and 4).

So far we have not had rabies produced by fresh brains showing no Negri bodies and no suspicious forms, but a few observers have claimed that such material has produced the disease. Therefore, until we can standardize our technic, we must in all such cases use animal inoculations. We may, however, be reasonably certain that a case showing such negative material was not a case of rabies. We may summarize our knowledge in regard to the worth of the smear method in diagnosis as follows:

1. Negri bodies demonstrated, diagnosis rabies.
2. Negri bodies and suspicious bodies not demonstrated in fresh brains, not rabies.
3. Negri bodies not demonstrated in decomposing brains, uncertain.
4. Suspicious bodies in fresh brains, probably rabies.

[1] Presse Méd., 1914, p. 79.

[2] Brit. Med. Jour., 1919.

[3] Arkwright: Brit. Med. Jour., 1919, June 23.

The *localization* of the Negri bodies is an important point in making diagnosis. We have found well-developed bodies distinctly localized in different parts of the brain in several instances. In horses there may be small, widely scattered areas of well-structured forms throughout the cerebellum, while tiny, indefinite forms are scattered through the rest of the brain examined. In human brains well-developed forms are found in the corpus striatum and not in the rest of the brain. In several dogs the localization has also been marked.

**The Complement-binding Test in Rabies.**—This test has been tried by Heller (1907), Friedberger (1907), and Baroni (1908), with negative results. Berry[1] (1910), and Olmstead and Wilson, in 1916, in our Research Laboratory, went over this work thoroughly and obtained similar negative results.

**Effect of Chemical and Physical Agents on Rabic Virus.**—Rabic virus appears to become attenuated under certain conditions of temperature; indeed, if it be subjected for about an hour to 50° C. or for half an hour to 60° C., its activity is completely destroyed. A 5 per cent. solution of carbolic acid, acting for the same period, exerts a similar effect, as do likewise 1 to 1000 solutions of bichloride of mercury, acetic acid, or potassium permanganate. Cumming has shown that formalin is especially deleterious to the virus. The virus also rapidly loses its strength by exposure to air, especially in sunlight; when, however, protected from heat, light, and air it retains its virulence for a long period.

The virus is readily filtered through all grades of Berkefeld filters, and from the glands through the coarser Chamberland. Poor and Steinhardt[2] have shown that the filtered gland and brain virus seem to have similar characteristics.

**Pathogenesis.**—**Natural Infection.**—The disease occurs in nature among the following animals given in order of their frequency: dogs, cats, wolves, horses, cows, pigs, skunks, deer, and man; in fact, as all warm-blooded animals are more or less susceptible to inoculations, all may presumably contract the disease when an open wound is brought in contact with infectious material of a rabid animal.

Rabies occurs in almost all parts of the world. It is most common in Russia, France, Belgium, and Italy; it is not infrequent in Austria and in those parts of Germany bordering on Russia. In this hemisphere it is infrequent in Canada, but in the United States[3] the cases are increasing in numbers. In Mexico and South America it occurs occasionally; while in England, North Germany, Switzerland, Holland and Denmark, because of the enforced quarantine laws, and of the wise provision that all dogs shall be muzzled, it is extremely rare. Recently in England more cases have been reported, due it is thought, to infected dogs carried in by aeroplanes. In Australia it is unknown, probably because the law that every dog imported into the island must first undergo a six months' quarantine has always been enforced. Since 1915 it has decreased markedly in New York City due to the fact that the muzzling ordinance has been more enforced. Just now it seems to be slightly on the increase.

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[1] Jour. Exp. Med., 1910, xii, 338.

[2] Jour. Inf. Dis., 1913, xiii, 203.

[3] Kerr and Stimson: Jour. Am. Med. Assn., 1909, liii, 989.

In this connection the question as to how long the sputum of a rabid dog may remain virulent after it drops from the animal is an interesting one. A case came under our observation in 1906 which illustrates this point. A child of six years came down with typical rabies in a neighborhood where there had recently been several cases of canine rabies, but no history of a bite could be obtained. The parents were sure she had not been bitten. Six weeks before, however, the child had fallen in the street and cut her cheek severely on a jagged stone. The wound was cauterized and healed without further trouble. A mad dog had been on that street just before this occurred. It is reasonable to suppose that the stone had on it some of the sputum from that dog, and so the child was infected. Such a case would not occur very often, but the possibility should be considered.

In regard to the question as to whether the bite of apparently healthy animals may give the disease, it may be said that, judging from laboratory experiments, some animals may have a light attack of the disease and recover spontaneously; though such cases, if they occur, are probably extremely rare. That the bite of an infected animal may give the disease before that animal shows symptoms has been proved. Fifteen days is the longest time reported between a bite and the appearance of symptoms in the dog. Therefore, if an animal is kept under observation three weeks after biting another, without developing symptoms, he may be pronounced free from suspicion.

Neither age, sex, nor occupation has any specific effect. The time of the year seems to have little effect, though most cases are said to occur during the summer months. The numbers vary with different years.

The certainty with which the disease may be produced after a bite and the rapidity of its development have been found to be governed by three factors: (1) the quantity of the rabic virus introduced; (2) the point of inoculation; (3) the strength of the virus as determined by the kind of animal which affords the cultivation ground for the growth of the organism. It is a matter of common observation that in man slight wounds of the skin of the limbs and of the back or wherever the skin is thick and the nerves few either produce no results, especially when bites are made through clothes, or are followed by the disease after an extremely long period of incubation; while in lacerated wounds of the tip of the fingers where small nerves are numerous or where the muscles and nerve trunks are reached, or in lacerated wounds of the face where there is also an abundance of nerves the period of incubation is usually much shorter and the disease generally more virulent.

These facts explain why only about 16 per cent. of human beings bitten by rabid animals and untreated appear to contract hydrophobia.

Since the establishment of the Pasteur treatment for the disease, the percentage of developed cases after bites is very much less—a fraction of 1 per cent.

**Symptoms.**—There is always a decided incubation period after the bite which varies within quite wide limits, but in the majority of cases it is from twenty to sixty days. Any period after six months is an exception; the shortest we have on record is fourteen days and the longest authentic period is seven months.

A very few apparently authentic cases have been reported as developing in about one year, but reports of any time beyond this must be received with doubt.

The symptoms may be divided into three stages: (1) The prodromal or melancholic stage; (2) the excited or convulsive stage; and (3) the paralytic stage.

When the second stage is the most pronounced the disease is called furious or convulsive rabies; when this stage is very short or practically lacking and paralysis begins early, the disease is called dumb or paralytic rabies.

IN THE DOG.—The principal symptoms of each form may be summarized as follows: (*a*) *Furious rabies:* change of behavior, biting (especially at those to whom the animal has been affectionate before), increased aggressiveness, characteristic restlessness, loss of appetite for ordinary food, with desire to eat unusual things, intermittent disturbance of consciousness, paroxysms of fury, peculiar howling bark, rapid emaciation, paralysis, beginning in the hindlimbs, death in great majority of cases in three to six days (exceptionally slightly longer) after the beginning of symptoms. (*b*) *Paralytic rabies:* short period of excitation, paralysis of the lower jaw, hoarse bark, appetite and consciousness disturbed, weakness, with paralysis spreading in great majority of cases, and death four or five days after first symptoms. There may be a number of cases showing transition types between these two forms.

IN HUMAN BEINGS.—*Furious Rabies.*—The first definite symptoms are difficult and gasping breath with a feeling of oppression and difficulty in swallowing, the latter the most characteristic symptom. It is caused by convulsive contraction of the throat muscles. The attacks are brought out when attempting to drink or swallow. The very thought of drinking may bring one on; and though there is no fear of water itself, there is fear of taking it because of the effect it produces. The convulsive attacks finally become more or less general over the whole body; in certain cases some parts are more affected by reflex excitation than others; for instance, there may be slight or no photophobia, while in exceptional cases, more frequently in dogs, the hydrophobia is also absent.

Most of the special reflexes are increased. Pupils become irregularly contracted and widened until they finally remain fixed.

Human beings are seldom dangerous to the people about them. In their convulsions they may bite things placed between their teeth, but not otherwise. At this time there is an increased flow of saliva, and one should avoid the contact with this in opened wounds. It may be so increased that the patient may try to get rid of it by taking it from the mouth with the hand and throwing it about. As a general thing, however, the patient has full possession of his senses between the convulsive attacks until very late in the disease.

The temperature is increased from 38° to 40° C., at first with morning remissions. Just before death it may rise as high as 42.8° C. (In lower animals the temperature sinks below normal just before death.) The pulse is generally over 100 and is irregular. This stage lasts from one to four days. Death may occur during a convulsion, but more often there is a *paralytic stage,* which lasts from two to eighteen hours. The convulsions become less frequent and the patient becomes weaker until finally there is a complete paralysis. At the beginning of this stage the patient may be able to drink water better than formerly. Death may occur at any time through paralysis of the heart or respiratory center.

*Paralytic Rabies.*—This form occurs quite seldom in human beings, more frequently in dogs, but not so often as the convulsive form. It is supposed to occur in humans and dogs after a more severe infection. Instead of periods of convulsions, the various muscles simply tremble and become gradually weaker until complete general paralysis supervenes. Sometimes paralysis develops very quickly and may be general before death from syncope or asphyxia occurs. This form generally lasts longer than ordinary rabies. Between these two typical forms of rabies there are many different types, giving quite varied pictures of the disease.

**Length of the Disease.**—The majority of the cases of furious rabies die on the third or fourth day after the symptoms show themselves. The limits of the reported cases are one to fifteen days, though there are reports of only one or two cases dying on any day after the ninth to the fifteenth. As the time when the symptoms really begin is difficult to notice, these statistics are probably only approximately correct. In paralytic rabies the average time in which death occurs is five days.

**Treatment.**—The old treatment of rabies consisted simply in encouraging bleeding from the wound, or in first excising the wound and then encouraging bleeding by means of ligatures, warm bathing, cupping-glasses, etc.; the raw surface was then freely cauterized with caustic potash, nitric acid, or the actual cautery. It is doubtful whether the disease ever manifested itself after such heroic treatment if the wounds were small and the treatment was begun soon after the bite; but when the wounds were numerous or extensive the mortality was still high. As it was often impossible to apply cauterization to the wound rapidly or deeply enough to insure complete destruction of the virus, Pasteur and others were led to study the disease experimentally in animals with the hope of finding some means of immunization or even cure. These investigations finally resulted in the discovery of methods of preventive inoculation applicable to man.

**Pasteur's Method of Preventive Inoculation.**—Pasteur's treatment is based upon the fact that rabic virus may be attentuated or intensified under certain conditions. He first observed that the tissues and fluids taken from rabid animals varied considerably in their virulence. Then he showed that the virus may be intensified by successive passage through certain animals (rabbits, guinea-pigs, cats) and weakened in passing through others (monkeys). If successive inoculations be made into rabbits with virus, either from the dog or the monkey, the virulence may be so exalted beyond that of the virus taken from a street dog, in which the incubation period is from twelve to fourteen days, that at the end of the fiftieth passage the incubation period may be reduced to about six or seven days when it remains fixed. This "*fixed virus*" was used by Pasteur and those after him in his preventive treatment because the dose could be more definitely regulated by subsequent attenuation or dilution.

*Original Method.*—A series of spinal cords from rabbits dead from "fixed virus" infection are cut into segments and suspended in sterile glass flasks plugged with cotton stoppers and containing a quantity of some hygroscopic material, such as caustic potash; these are kept at a temperature of about 22° C. The cord when taken out at the end of the first twenty-four hours is found to be almost as active as the fresh untreated cord; that removed at the end of forty-eight hours is slightly less active than that removed twenty-four hours previously; and the diminution in virulence, though gradual, progresses regularly and surely until, at the end of the eighth day the virus is inactive. Pasteur began his treatment with an emulsion of the cord kept until the fourteenth day. A certain quantity of this was injected into the animal that had been bitten; this was followed by an injection of an emulsion of a twelve-day cord; and so on until the animal had been injected with a perfectly fresh and therefore extremely active cord, corresponding to the fixed virus. Animals treated in this way were found by Pasteur to be absolutely protected, even against subdural inoculation with considerable quantities of the most virulent virus, and thus Pasteur's protective inoculation against rabies became an accomplished fact. As it would be undesirable to inject any but persons who had actually been bitten by a rabid, or presumably rabid animal, Pasteur continued his experiments in order to see whether it would not be possible to cure a patient already bitten. He carried on, therefore, a series of experiments which led to the discovery that if the process of inoculation be begun within five days of the bite in animals in which the incubation period was at least fourteen days, almost every animal bitten can be saved; and that even if the treatment be commenced at a longer interval after the bite a certain proportion of recoveries can be obtained. Thus the application of this method of treatment to the human subject was not tried until it had been proved in animals that such protection could be obtained and that such protection would last for at least one year and probably longer.

The chance of success in the human subject appears to be even greater than in the dog or rabbit. Man's period of incubation is comparatively prolonged. Thus there is an opportunity of obtaining immunity by beginning the process of vaccination soon after the bite has been inflicted, the protection being complete before the incubation period has passed.

**Present Administration of Pasteur's Treatment in Human Beings.**—The original method of Pasteur in its entirety was soon adopted in many lands, and his results were corroborated. Before long, however, a number of modifications were suggested by different observers, some slight, others more fundamental. Some have been widely used, such as Högyes' dilution method;[1] others have had a limited application in lower animals and are probably only of theoretic interest as regards man. Such are the intravenous inoculation of brain emulsions from street rabies into herbivora (Nocard and Roux, Protopopoff), and the intraperitoneal inoculations of large doses of fully virulent fixed virus into dogs, cats, or rabbits (Hellman, Heim, Remlinger). Immunity has been produced also in rats by allowing them to feed on rabid brains (Fermi, Repetto, Remlinger).

Högyes in Budapest was one of the first to use a different procedure. He claimed that the virus by Pasteur's method was attenuated only through the death of some of the specific organisms, that is, that there were simply fewer living organisms in the early doses given than in the later, and that therefore the same result might be obtained perhaps with even more accurate dosage by giving gradually decreasing dilutions of a fresh virulent cord. By diluting sufficiently he obtained a mixture which when inoculated did not produce rabies in the test animals, a result similar to that following an 8- to 10-day dried cord. This dilution he used for the first inoculation and gradually stronger dilutions for the succeeding ones.

Other methods of attenuating or diluting fixed virus have been used, such as exposure to the action of heat, cold, gastric juice, glycerin, or carbolic acid.

The mixed treatment with specific serum and vaccine has also been employed, chiefly by Marie,[2] by Remlinger, and by Babes.

**Methods of Attenuation by Gradual Drying.**—Pasteur's classic method has undergone modifications in three general directions: (1) lengthening or shortening the period of treatment; (2) starting the inoculations with a less attenuated cord; (3) increasing or decreasing the amount given at each injection. The method of drying the cords, however, has remained essentially the same as that used by Pasteur.

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[1] Nothnagel's Specielle Path. u. Ther., Wien, 1897.

[2] L'Etude expérimentale da la Rage, Paris, 1909.

The cord is removed by a modification of the method of Oshida in the following manner: Strict asepsis is preserved. The rabbit when completely paralyzed (seventh day) is killed by gas or chloroform and is dropped into a 5 per cent. solution of carbolic acid for five minutes. It is then removed, the excess of carbolic solution is drained off, and an incision through the skin at the upper and inner part of the thigh is made. The skin is loosened by cutting around the lower portion of the trunk. It is then pulled by the hands toward the upper extremity of the animal and over the head to the ears, leaving the back exposed and sterile throughout the entire length of the spine. The spine is then divided transversely near each extremity by bone-cutting forceps. The muscles are cut through about these areas so the spine may be more easily reached. With a long wire probe swabbed with cotton at one end the cord is pushed upward from its canal, freed from its nerves and membranes. The spine is steadied by lion-jawed forceps. The cord curls in a spiral as it emerges and rests on the sterile muscles of the neck. It is lifted with forceps, placed in a Petri dish and cut in two. A small piece is cut from one end and is dropped into a tube of broth to test its purity. A ligature with one long end is placed about each piece, both of which are then hung in a drying bottle (Fig. 203).

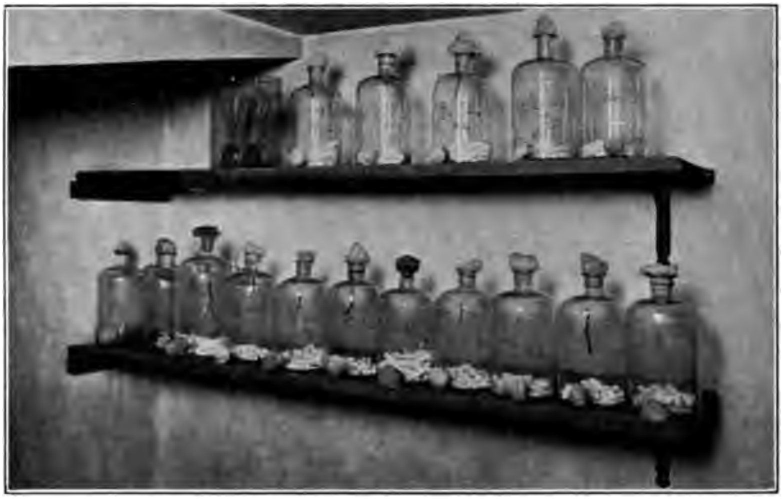

FIG. 203.—One corner of constant temperature room showing drying bottle containing fixed virus cords being prepared for vaccine.

DRYING THE CORD.—The drying bottles are sterile aspiration bottles with both openings plugged with cotton. A layer 1 inch high of sticks of caustic potash covers the bottom, and the pieces of cord are suspended from the top cotton plug by their attached ligatures. The bottles are then labelled and placed in the constant temperature room (Fig. 203) or incubator, which is kept at a temperature of about 22° C. (70° F.). After twenty-four hours' drying the cord is known as one-day cord; after two days, two-day cord, etc. Pieces of cord cut off at any time and put into glycerin will retain about the same strength for several weeks. This procedure is followed in regions where there are few cases of rabies, and the daily killing of rabbits to keep up the vaccine would be a large expense. It may also be followed where treatment is sent by mail.

The New York City Health Department used Pasteur's first schemata, with modifications, up to January, 1906, when they began treatment with a ten- and nine-day cord and finished with a one-day. They continued with this until August, 1913. Since then they have been using the more intensive method of the Hygienic Laboratory at Washington. From 1906 to 1913 inclusive they treated 4282 cases infected by rabid animals, with a total mortality of 0.54 per cent. and a corrected mortality of 0.19 per cent. They have had 7 cases of definite paraylsis with 2 deaths; 6850 cases in all, including those not bitten by rabid animals, were treated.

Since it had been found that fresh rabbit-fixed virus inoculated subcutaneously into man is apparently harmless, the Berlin Institute, with the hope of obtaining an earlier immunization and a shorter treatment, began to give still earlier cords. In 1901 it began with the eight-day cord on the first inoculation, and was inoculating a two-day cord on the eighth day of treatment. Its treatment lasted twenty-one days. This method was adopted at the Hygienic Laboratory in Washington in 1908, with slight variations for the different degrees of bites. Now only the intensive schema is used for all cases as follows:

TWENTY-ONE DAY SCHEME OF PASTEUR TREATMENT.

| Day. | Days cord. | Dried. | Number of injections. | Adults. | 6 to 10 yrs. | 1 to 5 yrs. |
|---|---|---|---|---|---|---|
| | | Face cases. | | | | |
| 1 . . | 8, 7 and 6 | 8 + 7 + 6 | 2 | 3 c.c. | 3 c.c. | 3 c.c. |
| 2 . . | 8, 7 and 6 | 8 + 7 + 6 | 2 | 3 " | 3 " | 3 " |
| 3 . . | 5 and 4 | 5 + 4 | 2 | 3 " | 3 " | 3 " |
| 4 . . | 5 | 5 | 1 | 2 " | 2 " | 2 " |
| 5 . . | 4 | 4 | 1 | 2 " | 2 " | 1½ " * |
| 6 . . | 4 | 4 | 1 | 2 " | 2 " | 1½ " * |
| 7 . . | 3 | 3 | 1 | 2 " | 1½ " * | 1 " * |
| 8 . . | 3 | 3 | 1 | 2 " | 1½ " * | 1 " * |
| 9 . . | 5 | 2 | 1 | 2 " | 2 " | 2 " |
| 10 . . | 4 | 4 | 1 | 2 " | 2 " | 2 " |
| 11 . . | 4 | 4 | 1 | 2 " | 2 " | 2 " |
| 12 . . | 3 | 3 | 1 | 2 " | 2 " | 1½ " * |
| 13 . . | 3 | 2 | 1 | 2 " | 2 " | 1½ " * |
| 14 . . | 4 | 4 | 1 | 2 " | 2 " | 2 " |
| 15 . . | 4 | 4 | 1 | 2 " | 2 " | 2 " |
| 16 . . | 3 | 3 | 1 | 2 " | 2 " | 1½ " * |
| 17 . . | 3 | 2 | 1 | 2 " | 2 " | 1½ " * |
| 18 . . | 4 | 4 | 1 | 2 " | 2 " | 2 " |
| 19 . . | 4 | 4 | 1 | 2 " | 2 " | 2 " |
| 20 . . | 3 | 3 | 1 | 2 " | 2 " | 2 " |
| 21 . . | 3 | 2 | 1 | 2 " | 2 " | 2 " |

0.1 per cent. phenol used as preservative instead of glycerin.
*Face cases receive adult doses.

Each of the first 3 doses contains a total of 1 cm. of the indicated cords. The succeeding doses each contain 0.33⅓ cm.

The New York City Health Department has been using this schema since May 7, 1919, for all cases, but it reduces all doses by one-fifth. In cases with very slight wounds which have begun treatment immediately the inoculations may be carried only as far as the fifteenth day.

The inoculations are made subcutaneously usually over the abdomen.

**Treatment by Mail.**—The New York City Health Department was the first to send out treatment by mail to physicians for their own patients. Full directions are sent with the mailing case. Two-tenths per cent. of carbolic acid is added to the emulsions prepared as above as a preservative.

The results from the treatment sent in this way seem to be equally as good as those from the treatment administered at the laboratory.

**More Intensive Treatment.**—In Berlin, where intensive treatment has been longest used, they began to employ even fresher cords for beginning doses because they continued to have late deaths, though not quite so often, after the more intensive methods they were using. Since 1910 the Pasteur Institute in Berlin has been using the following schema:

| Days | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age of cords | 3 | 2 | 1 | 1 | 3 | 2 | 1 | 1 | 3 | 2 | 1 | 1 | 3 | 2 | 1 | 1 | 3 | 2 | 1 | 1 | 1 |

The dose is 2 c.c. of cord emulsion (1 part of cord in 5 parts of sterile physiological salt solution) inoculated once a day into the subcutaneous tissue of the abdomen. Children and adults receive the same dose.

Simon gives the following statistics of the results of Berlin's increasingly intensive methods:

BERLIN STATISTICS.

| Period. | Age of cord used for beginning inoculation. | Cases. | Paralysis. | Mortality. | Per cent. |
|---|---|---|---|---|---|
| I. 1898-1906 | Chiefly 8-day cord | 2896 | 0 | 21 | 0.7 |
| II. 1906-1909 | 4-day cord. Sometimes 3-day cord | 1490 | 2 | 7 | 0.47 |
| III. 1909-1910 | 3-day cord for all cases | 819 | 3 | 5 | 0.6 |

Several other institutes are employing very intensive treatments, but their cases are still too few for consideration. Other directors still use the older methods.

**Rapid Drying of Rabies Virus.**—Harris, of St. Louis, has published a new method of drying rabies virus and of regulating the dosage.

TECHNIC.—The brain and cord are removed aseptically and ground up in a sterile mortar with a sufficient quantity of $CO_2$ snow thoroughly to freeze the tissue. The frozen nerve tissue and snow are then placed in a Scheibler jar over $H_2SO_4$, the jar being kept in a Frigo apparatus. A vacuum of from 5 to 2 mm. is produced in the jar, which is then kept at the temperature of 18° C. by an ice and salt mixture for a sufficient length of time to dry thoroughly the nerve substance, which then appears as a dry powder. About two days are required for one brain and cord, which lose about one-half of their virulence in the process. The powder is then sealed in tubes *in vacuo* and kept at a temperature below 0° C. until required for use. It has been found that by keeping the powder thoroughly dry and cold practically no further loss of virulence occurs for at least six months. Before storing the virus for use its strength in units is computed, the unit being the minimal infecting dose (M. I. D.) for a rabbit when injected intracerebrally.

The advantages claimed for this method are: (1) the ease and economy with which a large amount of virus can be prepared, it being necessary to prepare the virus for use even in large laboratories only at intervals of several months; (2) the possibility of more accurate dosage for the patients; (3) a shortened period of treatment; and (4) the inoculation of more virus units. The required amount of powdered virus is weighed out each morning, and the necessary dilutions in salt solution for the various patients are made from this.

Up to October 13, 1913, Harris had treated 240 cases exposed to infection from dogs, in which the diagnosis of rabies was either proved by laboratory methods or strongly probable from veterinary diagnosis. Of this number one patient, who had started treatment six days after the bite, died of rabies during the period of observation. No cases of paralysis have been reported.

We must wait for further statistics before being able to judge of the efficiency of this method.

FIXED VIRUS MODIFIED BY DIALYSIS.—Cumming, of Ann Arbor, has devised a method of antirabic vaccination, by which he uses fixed virus which has been rendered avirulent by dialysis.

The emulsion of fixed virus is placed in collodion sacs (prepared by the Novy method and sterilized in the autoclave at 105° C. for twenty minutes) and dialyzed in distilled water for from twelve to twenty-four hours. The resulting vaccine does not produce rabies on intracranial inoculations, but does produce immunity on subcutaneous inoculations. Experiments by Cumming on rabbits show that whereas the original Pasteur method protects against only twice the minimum lethal dose (minute directions for obtaining the M. L. D. are given) injected intracerebrally, and the Högyes method against one and one-half times the fatal dose, the dialysis method protects against at least three times the fatal dose. He also claims that immunity is produced at an earlier date than by the other methods. Treatment (2 c.c. of the vaccine) is given daily for from fifteen to twenty-five days. Cumming reports over 800 cases (62 per cent. bitten by animals proved to have been rabid) treated without a death and without complications.

We must wait for further practical testing of this interesting method before deciding as to its comparative worth.

**Marie's Method.**—For several years past the use of virus serum mixture has been in vogue at the Pasteur Institute in Paris, the technic of which is as follows:

1 gm. of the medulla of a rabbit dead of fixed virus is finely emulsified with 9 c.c. of 0.8 per cent. salt solution and filtered through linen. Two c.c. of this emulsion and 4 c.c. of antirabic serum (obtained from sheep, and inactivated at 56° C. for thirty minutes) are carefully mixed after standing for a time. Six c.c. of this mixture, which contains an excess of virus is injected into the patient. These injections are repeated on the next three days, after which the treatment proceeds according to the regular Pasteur schema, beginning with the use of a six-day cord on the fifth day. The antirabic serum is obtained from sheep which have been subjected to a long and strong course of treatment with fixed virus. It is claimed that a quicker immunity is produced by the serum-virus mixture than by the original Pasteur schema, an advantage of especial value in the treatment of cases liable to become infected with a short incubation, such as bites on the head.

**Antirabic Serum.**—The possibility that the serum of animals immunized against rabies contains protective substances was suggested by Pasteur as early as 1889. The following year Babes recommended the use of the serum of vaccinated animals in combination with the Pasteur treatment. Since then the study of the amount and character of the antibody content of animals immunized against rabies has been carried on more or less extensively both from the theoretic and the practical sides. It was hoped that a serum could be obtained that would effect a cure for developed rabies just as diphtheria antitoxin does for developed diphtheria. But such a definite applicability of the serum has not developed. It was soon found that, while serum of certain vaccinated animals possessed the property of neutralizing rabies virus *in vitro,* it had only a slight inhibiting power when inoculated into the living animal, and apparently no action at all by any method of inoculation after the disease had become manifest. Babes still claims, however, that the serum has enough effect *in vivo* to be used in treatment, and his serum treatment is based upon this claim. He gives as his reason for employing serum at the end of treatment that he wishes to introduce into the patient at the time he most needs it the largest amount of antibodies. He also claims that the serum so given will prevent or cure the occasional paralyses which occur during treatment.

Those who did not agree with Babes were led to test the practical use of the serum combined with the beginning vaccine inoculations.

Remlinger, Marie, and others showed that a serum-virus mixture with a slight excess of virus will protect an animal against infection into the anterior chamber of the eye when inoculated during the three days following the vaccination. Thus Marie showed that immunity is produced more quickly by these unsaturated mixtures of virus and serum than by the virus alone. If a surplus of serum is present the animals are not protected from a later infection.

Marie, who has used the serum in his treatment of humans since 1904, prepares it as follows:

The brains of two rabbits dying from fixed virus infection are finely rubbed up with physiologic salt solution in the proportion of 20 gm. in 180 c.c. This emulsion is filtered through fine cloth and heated for one-half hour at 37° C. Sheep are used for the inoculation. Each sheep receives intravenously 30 c.c. (3 gm. fixed virus) a week for six to eight weeks. Thirteen days after the last inoculation the first blood is drawn. Then in a period of two weeks, at 4 bleedings, 200 c.c. of blood are drawn. After a fourteen-day pause another series of inoculations are given and the animal is ready for another series of bleedings. From each animal yearly about 3 liters of antirabic serum are obtained. A strong serum is one that neutralizes 40 virus units in 1 c.c.

A *virus unit* is 1 c.c. of five times the dilution of fixed virus that will surely kill a rabbit inoculated intracerebrally, *e. g.*, the unit of a fixed virus that will surely kill a rabbit in 1 to 500 dilution is 1 c.c. of a 1 to 100 dilution.

The nature of the antibodies in rabies serum has been the subject of many studies. Fermi and a few others claim that the antibodies are not specific. They say that they can obtain a similar serum after the inoculation of normal brain emulsions. Some even use normal brain emulsions in the treatment of their lighter cases.

Certain investigators (Kraus, Marie, and others), while not able to corroborate all of these claims, have found that the sera of certain animals which are more or less refractory to rabies possess a small amount of rabicidal strength; *e. g.*, 0.5 c.c. of normal chicken serum mixed with one unit of fixed virus (1 c.c. of 1 to 100 dilution) causes the latter to become neutral in eighteen hours.

All species of animals tried produce the specific antibodies, but not to an equal degree. Human beings and monkeys are said to have more antibodies after vaccination than rabbits.

Centanni showed that immediately after vaccination the animal is not fully protected, though its serum may contain antirabic qualities while later the animal is immune, though its serum may not be able to neutralize the rabies virus. These facts point to a cellular immunity.

**Results of Antirabic Treatment.**—On the whole the results of protective inoculations against rabies are marked. One has only to compare, the statistics of mortality after bites from animals suffering from hydrophobia with those given after any of the methods of treatment employed to see the benefit. As regards the best method to use, the case is different. With many methods tried in many lands on a large number of cases, it would seem that we should be able by this time to determine their comparative worth. But the trouble is that the improvement on the whole is not great and the statistics are not kept uniformly or minutely enough to draw trustworthy comparisons.

A slight decrease in mortality has been shown in the statistics from most of the antirabic institutes of the world.

But these figures tell us little about the actual value of the different methods. In order to be able better to judge, the statistics should uniformly give many more details. Some institutes give such details, others do not. Until some such scheme as the following is carried out by all, we must change cautiously a treatment that has already given good results.

1. Diagnosis of biting animal:
   (*a*) Rabies, (*b*) probably rabies, (*c*) questionable, (*d*) not rabies, (*e*) nothing known.

2. Manner of making diagnosis:
    (*a*) By animal inoculation, (*b*) by microscopic examination, (*c*) by clinical diagnosis.
3. Site and character of bites (*e. g.*, number, depth, laceration, protected by clothing, etc.):
    (*a*) Head, (*b*) hands, (*c*) other parts of body.
4. Time elapsing between bite and beginning of treatment.
5. Method of treatment used.
6. Complications during or after treatment, particularly paralysis.
7. Character and time of death.

That the time after the bite makes a great difference is shown by the following table:

| Time intervening between bite and beginning treatment | | Number of cases treated. | Death. | Percentages. |
|---|---|---|---|---|
| Babes | 1 to 2 days | 3406 | 3 | 0.088 |
| | 3 to 5 days | 2541 | 2 | 0.077 |
| | 5 to 6 days | 809 | 1 | 0.124 |
| Diatroptoff | 1 week | 4602 | 26 | 0.560 |
| | 2 weeks | 961 | 16 | 1.660 |
| | 3 weeks | 313 | 10 | 3.190 |

IMMUNITY.—The immunity in human beings produced by the antirabic treatment apparently lasts a variable time. That it may not last more than fourteen months is shown by the history of one of our cases. The patient was an assistant in a hospital for dogs. He was given eighteen days' treatment after a light wound in the hand from a rabid dog. Fourteen months later he came down with typical hydrophobia. Since his treatment he had become very careless with cases of rabies, exposing wounded hands to saliva because he considered himself immune. He was warned that there might be danger. Six weeks before his death he put a wounded hand into the mouth of a rabid animal. There is little doubt that this is a case of reinfection after loss of protection from the treatment rather than one of delayed hydrophobia.

Marie has found complete immunity in dogs eighteen months after treatment.

**Ill Effects of Treatment.**—LOCAL.—There is only slight local discomfort, increased a little if the emulsion contains glycerin. During the second week an erythema often appears about the point of inoculation, which Stimson regards as a manifestation of hypersusceptibility to foreign nerve tissue. It disappears in a few days.

CONSTITUTIONAL.—Ever since the beginning of treatment occasional non-fatal affections of the nervous system have been reported, which occurred during or shortly after the course of treatment. These have varied in degree all the way from a slight neuritis, through paraplegias to paralyses of various parts of the body. Very occasionally the paralyses are marked and the patient dies. Cases of true paralytic rabies which may occur within the period required for the establishment of immunity by the treatment must be differentiated from cases occurring as a result of treatment.

Simon published an extensive report of 84 cases of paralysis occurring during the years 1888 to 1911 inclusive, and Fielder[1] gave a corroborative report in 1916. Simon classifies the cases collected by him according to the diagnosis of the biting animal, with the mortality in each group as follows:

| Positive group. | | Probable group. | | Questionable group. | | Negative group. | | Not known group. | |
|---|---|---|---|---|---|---|---|---|---|
| Number cases. | Per cent. | Number cases. | Per cent. | Number cases. | Per cent. | Number cases. | Per cent. | Number cases. | Per cent. |
| 25 | 29.76 | 11 | 13.0 | 21 | 25 | 17 | 20.23 | 10 | 11.9 |
| (2) | | (4) | | (5) | | (3) | | (5) | |

Nineteen deaths occurred, as seen from the figures in parentheses, or 22 per cent. of the 84 cases.

In analyzing the effect of different methods of treatment on paralyses, Simon gives the following summary:

| | Number of cases treated. | Cases of paralysis. | Proportion. |
|---|---|---|---|
| Classic Pasteur method . . . . | 32,676 | 6 | 1 in 5446 |
| Modified Pasteur method . . . . | 8,657 | 16 | 1 in 541 |
| Högyes method . . . . . . . . | 51,417 | 3 | 1 in 17139 |

It is seen that the number of paralyses following the Höyges method are markedly less than those following the other methods.

From the studies so far made of these paralyses the possibility of there being different causes for different cases cannot yet be ruled out. The chief theories advanced as to factors in producing the condition are six:

1. Due to "laboratory rabies" from the fixed virus vaccine inoculated.
2. Due to "modified rabies" resulting from the treatment on the street virus infection.
3. Due to a toxin produced by the rabies organisms.
4. Due to infection with extraneous organisms introduced with the virus during treatment.
5. Due to psychological disorders.
6. Due to the inoculation of a foreign protein followed by an anaphylactic reaction.

**The Cauterization of Infected Wounds.**—We believe that in cases in which the Pasteur treatment cannot be applied great benefit may be derived from the correct use of cauterization with *fuming nitric acid*, even forty-eight hours after infection, and that even in cases in which the Pasteur treatment can be given, an *early* cauterization will be of great assistance as a routine practice and should be very valuable, as the Pasteur treatment is frequently delayed several days for obvious reasons, and then does not always protect. In the case of small wounds all the treatment probably indicated will be thorough cauterization with nitric acid within twelve hours from the time of infection. Our experience in dealing with those bitten by rabid animals goes to show that physicians do not appreciate the value of thorough cauterization of the infected wounds.

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[1] Jour. Am. Med. Assn., 1916, lxvi.

**Preventive Measures in Animals.**—Far more important than any treatment, curative or preventive, for hydrophobia in man is the prevention of rabies in dogs, through which this disease is usually conveyed. Were all dogs under legislative control and the compulsory wearing of muzzles rigidly enforced for two years where rabies prevails, hydrophobia would practically be stamped out. This fact has been amply demonstrated by the statistics of rabies in countries (*e. g.*, England) where such laws are in force. New York City has recently had some measure of success in enforcing such laws.

## YELLOW FEVER.

Yellow fever is an acute infectious disease of tropical countries, with no characteristic lesions except jaundice and hemorrhage. Other lesions that exist are those common to toxemia. One attack usually produces complete immunity.

**Historical Note.**—There have been many extensive studies on the etiology of this disease with numerous announcements of the discovery of its specific cause. Not one of the latter, however, has been corroborated. The Bacillus icteroides of Sanarelli (1897), found in the circulating blood and in the tissues of most yellow-fever patients, was thought by many to be the real organism, and for some time it was the subject of most minute studies with the result that it, too, has been placed with the rejected organisms.

The epoch-making investigations of the United States Army Commission composed of Walter Reed, James Carroll, Aristides Agramonte, and Jesse W. Lazear[1] (1901), established the truth, that this disease, like malaria, is carried from one infected person to another through the agency of a mosquito. Finley, in 1881, was the first positively to assert that the mosquito was the transmitter of the disease. He was, however, unable to prove his theory, and it remained for the commission conclusively to show that a distinct species of mosquito carried the infection.

The work of the American commission was fully corroborated by the French commission and by other workers.

The principal facts established by the commission have been summed up by Goldberger[2] as follows:

1. Yellow fever is transmitted, under natural conditions, only by the bite of a mosquito (*Aedes calopus*) that at least twelve days before has fed on the blood of a person sick with this disease during the first three days of his illness.
2. Yellow fever can be produced in man under artificial conditions by the subcutaneous injection of blood taken from the general circulation of a person sick with this disease during the first three days of his illness.
3. Yellow fever is not conveyed by fomites.
4. *Bacillus icteroides* Sanarelli stands in no causative relation to yellow fever.

Though the specific parasite remains yet undiscovered, facts have been brought out by these studies which give some idea of its character.

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[1] Jour. Exp. Med., 1900, v, 215; Jour. Am. Med. Assn., 1901, xxxvi, 413.

[2] The Yellow Fever Institute Bulletin No. 16 with review of literature to 1907.

1. It seems to require two hosts (a mammal and an arthropod) for the completion of its life cycle (analogies, *Plasmodium malariæ*, *Babesia bigeminum*).
2. There is a definite time after the bite of the mosquito before the blood of the person bitten becomes infective (average five days), and a definite time that the blood remains infective (three days).
3. The blood during these three days is still infective after passing through the finest-grained porcelain filters (Chamberland B and F).
4. The blood loses its virulence quickly (forty-eight hours) when exposed to the air at a temperature of 24° to 30° C. When protected from the air by oil and kept at the same temperature it remained virulent longer (five to eight days). Heated for five minutes at 55° C. it becomes non-virulent.
5. The bite of an infected mosquito does not become infectious until twelve days (at a temperature of 31° C.) after it has bitten the first patient.

The cause of the disease still remains undiscovered, notwithstanding much study of human blood and other tissues and of infected mosquitoes. The infective blood filtrates show nothing with the dark-field illumination except small dancing granules similar to those found in healthy persons.

Certain facts relating to the disease seem to point to protozoa as the cause; for instance, the necessity for a second host and the long incubation time required before that host becomes infective after biting a yellow-fever patient.

The bodies described under the name paraplasma have been shown by Agramonte to be tissue changes.

The higher monkeys seem to be susceptible, though no complete experiments have been made with them.

Noguchi[1] has just reported finding a spirochete, similar to that found in Weil's disease, in the blood of cases diagnosed as yellow fever in Quayagnit in 3 out of 11 cases. He first found the spirochete in the blood of guinea-pigs inoculated with the blood of patients in 6 out of 27 cases. Only 8 out of 74 guinea-pigs showed them. The infected guinea-pigs had all of the symptoms of the yellow fever cases. Noguchi has named this spirochete *Leptospira icteroides*. From the demonstration of antibodies in the patient's serum Noguchi concludes that probably the leptospira icteroides is etiologically related to yellow fever.

**The Yellow-fever Mosquito** (Figs. 204 to 209).—The name *Stegomyia* for this small tropical mosquito was suggested by the English entomologist Theobald, who separated this genus from the genus *Culex*, with which it was formerly classed. It was first given the specific name *Fasciata*, but Blanchard proved that this had already been used and the name *Calopus* (Meigen, 1818) was found to be the proper one. Later the genus *Stegomyia* was shown to be invalid and the organism now goes by the name *Aedes calopus* Meigen. Salient characteristics of *Aedes* are: (1) The palpi in the male are as long or nearly as long, as the proboscis; in the female the palpi are uniformly less than one-half as long; (2) the legs are destitute of erect scales; and are alternately banded white and black; (3) the thorax is marked with lines of silvery scales. *Aedes calopus* is spread over a wide range of territory, embracing many varieties of climate and natural conditions. It has been found as far north as Charleston, S. C., and as far south as Rio de la Plata. There is no reason to believe that it may not be present at some time or other in any of the intermediate countries. In the United States specimens of *Aedes calopus* have been captured in Georgia, Louisiana, South Carolina, and eastern Texas. The island of Cuba is overrun with this insect. The fact that *Aedes calopus* has been known to exist at various times in Spain and other European countries may account for the spread of yellow fever which has occurred there once or twice in former times; the same may be said of the country farther north in the United States, where *Aedes calopus* has not yet been reported, but which have suffered from invasions of yellow fever.

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[1] Jour. Exp. Med., 1919, xxix, 565 and xxx, 1, 9 and 13.

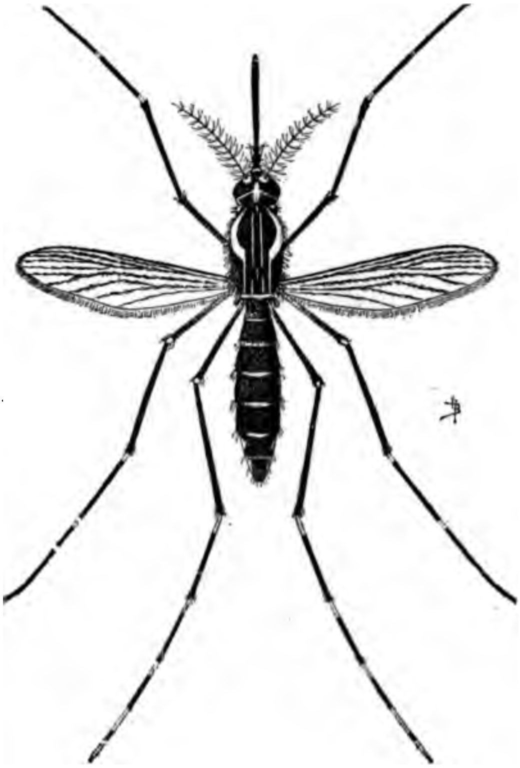

FIG. 204.—The yellow-fever mosquito (Aedes calopus). Adult female. Much enlarged. (Howard.[1])

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[1] Farmers' Bulletin, No. 547, U. S. Dept. of Agric., Washington, D. C.

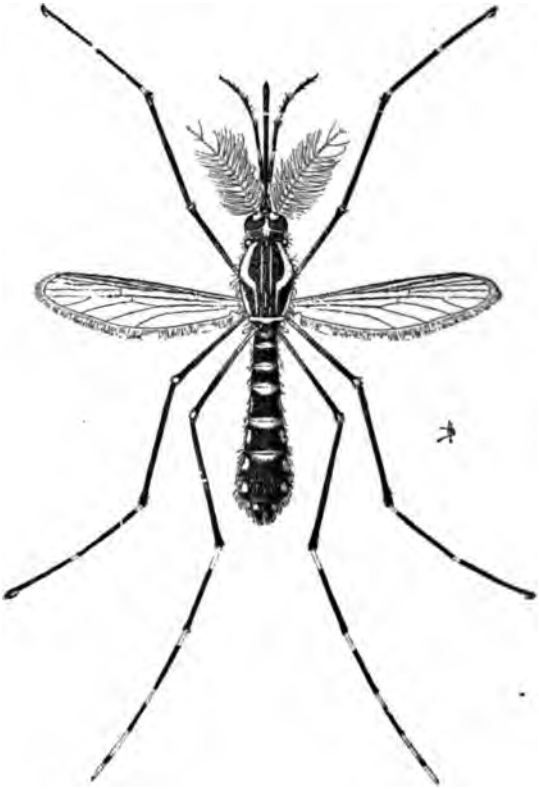

Fig. 205.—The yellow-fever mosquito. Adult male. Much enlarged. (Howard.)

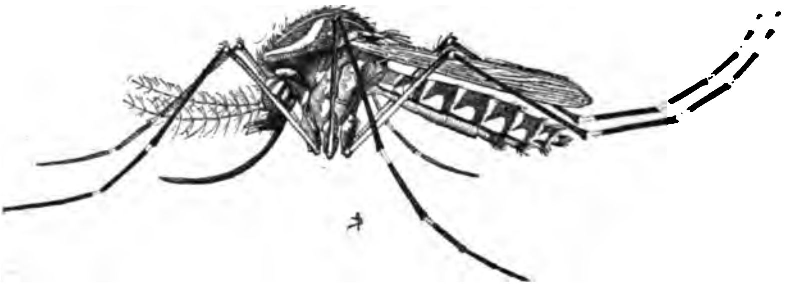

Fig. 206.—The yellow-fever mosquito. Adult female, side view. Much enlarged. (Howard.)

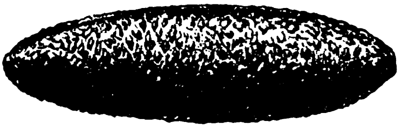

Fig. 207.—The yellow-fever mosquito. Egg. Greatly enlarged. (Howard.)

The adult mosquito is so small that mosquito netting of 19 or 20 strands or meshes to the inch is required to keep the insect from entering a place.

Brackish water is unsuited for the development of *Aedes* larvæ. The species *Aedes calopus* seems to select any deposit of water which is comparatively clean. The defective drains along the eaves of tile roofs are a favorite breeding place in Havana and its suburbs; indoors they find an excellent medium in the water of cups of tin or china into which the legs of tables are usually thrust to protect the contents from the invasion of ants, a veritable pest in tropical countries. The same may be said of shallow traps, where the water is not frequently disturbed.

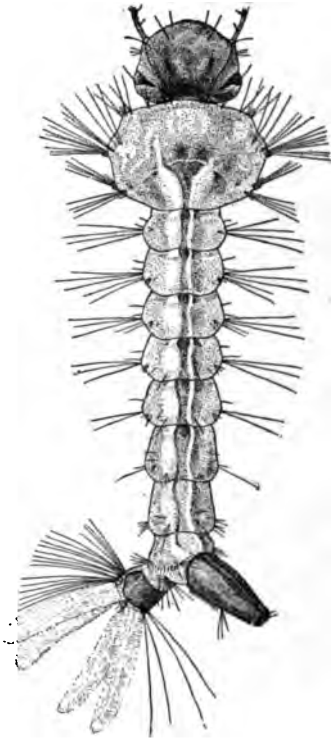

FIG. 208.—The yellow-fever mosquito. Larva. Much enlarged. (Howard.)

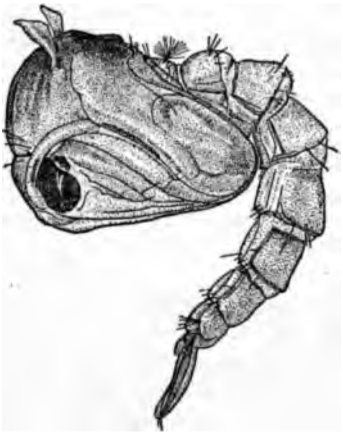

Fig. 209.—The yellow-fever mosquito. Pupa. Much enlarged. (Howard.)

Like other *Culicidæ*, it prefers to lay at night. It is eminently a town insect, seldom breeding far outside of the city limits. Agramonte never found *Aedes calopus* resting under bushes, in open fields, or in the woods; this fact explains the well-founded opinion that yellow fever is a domiciliary infection.

The question of hibernation in the larval stage is important. Agramonte failed to get larvæ that could resist freezing temperature, and found that in the case of *Aedes calopus* this degree of cold was invariably fatal.

The possibility of their being capable of life outside their natural element must also be considered from an epidemiological point of view. The dry season in the countries where this species seems to abound is never so prolonged as completely to dry up the usual breeding places. Experimentally, adult larvæ removed from the water and placed overnight upon moist filter paper could not be revived the following morning.

The question of the life period of the female insect is of the greatest importance when we come to consider the apparently long interval which at times has occurred between the stamping out of an epidemic of yellow fever and its new outbreak without introduction of new cases. The fact is that *Aedes calopus* is a long-lived insect; one individual was kept by Agramonte in a jar through March and April into May, in all for seventy-six days after hatching in the laboratory.

These mosquitoes bite principally in the late afternoon, though they may be incited to take blood at any hour of the day. They are abundant from March to September, and even in November Agramonte was able to capture them at will in his office and laboratory.

The mosquito is generally believed to be incapable of long flight unless very materially assisted by the wind. At any rate, the close study of the spread of infection of yellow fever shows that the tendency is for it to remain restricted within very limited areas, and that whenever it has travelled far beyond this, the means afforded (railway cars, vessels, etc.) have been other than the natural flight of the insect.

Experiments have demonstrated that not all mosquitoes which bite a yellow-fever patient become infected, but that of several which bite at the same time some may fail either to get the parasite or to allow its later development in their body. This condition is similar to that seen in *Anopheles*, with regard to malaria.

The question as to the length of time infected mosquitoes remain dangerous to the non-immune community cannot be definitely answered at present; there is good presumptive evidence that the mosquito may harbor the parasite through the winter and be enabled to transmit in the spring an infection acquired in the fall. There is reason to believe that the mosquito, once infected, can transmit the disease at any time during the remainder of its life. Freezing temperature, however, quickly kills the insect.

Carrying out preventive measures based on the knowledge gained by the splendid work of the American Army Commission, yellow fever has been practically wiped out of Cuba, the Isthmian Canal Zone, and other infected areas.

# PART III.

# APPLIED MICROBIOLOGY

## CHAPTER XLVI

## THE PRACTICAL APPLICATION OF BACTERIAL VACCINES.

THE practical application of bacterial vaccines for prophylactic purposes developed as a result of the success of vaccination against smallpox and the Pasteur prophylaxis in rabies. The first successful application was that of Pasteur who immunized sheep against anthrax by the injection of attenuated cultures. The protection conferred by the injection of bacterial vaccines is associated with a rise in the antibody content of the blood, and this increase has been assumed to be the basis of the protection. This increase may be only a part of the mechanism of protection, as the demonstrable antibodies disappear long before the immunity is lost. The view, however, that the protection was primarily due to the enhanced antibody content of the blood led to the therapeutic use of vaccines, with the hope that even during an infection they would stimulate further antibody production and thus be an aid in recovery.

The rational application of bacterial vaccines as specific prophylactic or therapeutic agents presupposes a correct bacteriológical diagnosis, or at least a clinical diagnosis which warrants the deduction that the infection is due to a specific bacterium. It must be kept in mind that many of our bacterial names refer not to a single organism but rather to species in which the members of one subgroup frequently have little, if any, immunological relationship with members of other subgroups, or the members of subgroups may be completely heterogeneous as regards such relationship. On the diagnosis and a knowledge of immunological characteristics of the causative organism depends the selection of the vaccine and whether an autogenous or a stock vaccine is applicable.

**Polyvalent Vaccines.**—Many of the so-called polyvalent vaccines are not polyvalent in the sense that they contain representatives based on immunological knowledge. In most instances they are mixtures of many strains under a group name.

**Vaccines of Mixtures of Bacteria, So-called Mixed Vaccines.**—One type is a shotgun preparation advocated where a bacteriological diagnosis is absent. Another type is a mixture of the organisms usually found associated in certain types of inflammation. Unfortunately the types found in such inflammation are usually members of heterogeneous groups so that the applicability of such stock vaccines as specific agents is more apparent than real. Mixed vaccines of specific types of bacilli for prophylactic purposes are discussed later.

**Types of Vaccines.—Killed Bacteria.**—This type of vaccine is the most commonly used. Heat is usually employed to kill the bacteria. The minimum temperature and time of exposure necessary to kill, should be used, as overheating may lower or destroy the antigenic value of the vaccine. Disinfectants such as carbolic, tricresol, chloroform, ether, etc., have been advised, but there is no definite evidence that they are superior to minimum heating. They are, however, often added as preservatives. Two types of vaccines are used, in one the menstruum is normal saline, in the other an oil. The latter is called *lipovaccine* (see below).

**Bacterial Extracts, Autolysates, Digested Bacteria.**—The object of such preparations is to hasten absorption or to bring into solution the toxic elements of the bacteria. In approximating these conditions, however, the reactions become severe or even serious and such preparations should be very cautiously employed.

**Live Bacteria.**—Their use is based on the fact that an unnatural portal of entry is associated with a local reaction only. The advantage would be that the unchanged bacteria should stimulate a higher immunity. There is always the possibility, however, that, should they accidentally reach the normal portal of entry, disease might follow. The use of vaccines of bacteria attenuated in virulence by various methods is common in veterinary medicine.

**Sensitized Vaccines.**—*Living or Killed.*—The bacteria are treated with immune sera to lessen or avoid the local and general reaction, Besredka and others[1] claiming that this will not interfere with their immunizing value. Larger and more frequent doses are thus possible and the sensitized bacteria are probably more quickly disintegrated and absorbed. The sensitized dead vaccines have been most commonly employed, and those left alive often die before use.

The superiority of sensitized over the ordinary killed vaccine has not been demonstrated. Animal experiments have shown the results of the two methods to be very similar. A similar procedure, viz., the simultaneous injection of vaccines and immune serum is employed in certain prophylactic procedures, where the bacterium employed gives rise to excessive reactions.

**Preparation of Bacterial Vaccines in Saline Solution.**—Cultures are preferably grown on agar, although broth may be used. The growth after twenty-four to forty-eight hours, depending on the rapidity of growth of the organism, is washed off in a small amount of saline solution. This suspension should be well shaken to give an even distribution of bacteria and is then standardized before heating or the addition of preservatives. The Wright method is most commonly used for standardization.

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[1] Gay and Claypoole: Arch. Int. Med., 1914, p. 671. Sawyer; Jour. Am. Med. Assn. 1915, lxv, 1413. Nichols: Jour. Exp. Med., 1915, xxii, 780,

A capillary pipette (see p. 220) is marked about one inch from the tip. The finger is pricked and blood drawn up to this mark; a bubble of air is then allowed to enter the tube and the bacterial suspension drawn up to the mark. The contents are then mixed on a slide and thin smears (as for blood) made and stained. On the under lens of the eye-piece a one-quarter-inch square is marked out with a pencil, and using an oil-immersion objective the number of bacteria and red cells appearing in this square are counted separately. About fifty squares are counted and the average per square obtained. The number of red cells per cubic centimeter being known, the number of bacteria are obtained according to the following proportion: Number of red cells per square : number of bacteria per square : : 5000 (millions) : X (millions).

Other methods are employed, as direct counting, either by the Prescott method, in a counting chamber as for blood cells or platelets, centrifuging and determining the volume of bacteria, or by determining the weight of the bacteria after evaporation and drying.

The suspension is heated to not over 55° C. for one hour unless the organism is not killed, and a preservative tricresol 0.3 per cent., carbolic acid 0.25 per cent. or lysol 0.25 per cent. added after making the necessary tests for sterility. For the American and English armies typhoid vaccine is only heated to 53° C. and the antiseptic relied on to finish sterilization. The vaccine is diluted for use with saline containing a preservative. The French add no antiseptic.

Sensitized vaccines are prepared by adding immune serum to the suspension and after several hours sedimenting the bacteria by means of the centrifuge and washing them free of serum with saline solution and suspending them finally in saline solution. Gay and Claypoole employ alcohol-killed sensitized vaccines.

**Preparation of Bacterial Vaccines in Oil Lipo-Vaccine.**—The method[1] employed by the Army is to grow the bacteria in broth or on agar, making saline suspensions from the latter. The bacteria are then collected by centrifuge. For large amounts a Sharples separator type of centrifuge is used. The bacterial mass in the latter case is scraped from the interior of the cylinder and placed in petri dishes. These are then placed in an oven over and under unslaked lime and heated to 53° C. for eighteen to twenty-four hours. The bacteria dry completely within a few hours, the continuation of the heating does not therefore injure the antigenic property. The dried mass is then collected and weighed. The appropriate amount of dried material is placed in a grinding jar containing about 70 steel balls of ½ inch and ¼ inch diameter, tightly corked and the jar placed in a revolving apparatus (Abbe ball mill) geared to revolve at 70 revolutions a minute. This grinding by the revolving steel balls is continued for four to twenty-four hours. Different workers are not agreed as to the minimum time. Excessive grinding means fragmentation of the bacilli which might be a factor in increasing reactions by increasing the rapidity of absorption. Add a mixture of two parts of cotton-seed oil (Wesson's) and one part of lanolin and grind again for twelve to sixteen hours. Then add oil to the final volume desired and sufficient chlorbutanol to give 0.5 per cent. and grind again for four to twelve hours. The volumes are adjusted so that the final content of lanolin is 2 per cent. The oil and lanolin are sterilized in the autoclave at 15 pounds' pressure for thirty minutes. All the steps in the preparation must be carried out with scrupulous attention to asepsis. Contamination is easy because of the many handlings. The dried powder should be tested for sterility as well as the end product. As the bacteria are dry, if not all are killed by the heating they may remain viable in the oil for long periods of time, in spite of the preservative added. The content in bacteria is standardized by weight, this having been arrived at by comparative counts and weights, as well as by test injections. The amount of the dose per cubic centimeter for prophylactic vaccines is given by the Army Medical School as follows:

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[1] Whitmore, Fennel and Peterson: Jour. Am. Med. Assn., 1918, lxx, 427. Fennel: Jour. Am. Med. Assn., 1918, lxxi, 2115. Rosenow: Jour. Am. Med. Assn., 1919.

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|---|---|
| Pneumococcus (Types I, II and III, equal parts) | 2.5 to 3.5 mgm. |
| Triple vaccine (B. typhosus, B. paratyphosus A and B, equal parts) | 0.9 " |
| Meningococcus | 1.8 " |
| Cholera | 1.5 " |
| Plague | 1.2 " |
| Dysentery (not available). | |

Rosenau has devised a ingenious method for drying the bacilli. He collects the bacilli as above but suspends them again in a minimum of water. This is then transferred to a heavy wall flask containing glass beads and for each c.c. there is added 5 c.c. of oil. This is then connected with a pump (Gerrick type) with an appropriate tube-bulb connection to prevent boiling over. While exhausting the air the flask is kept on a water-bath at 60° C. and shaken every five or ten minutes. Because of the extreme vacuum the water boils away at this temperature. The operation takes about two to three hours. The content is standardized by count on the water suspension. Subsequent dilution is made by adding oil containing lanolin, the latter sufficient to give an end amount of 2 per cent.

**Relative Value of Saline and Lipo-Vaccines.**—We can only compare them in relation to prophylactic use. The underlying principle of the oil vaccine is that the bacilli being dried, autolysis is prevented, the products of which being absorbed rapidly cause severe reactions. The oil itself also delays the dissolution of the bacteria and their absorption. This results in gradual absorption or is equivalent to the injection of repeated small doses of saline vaccine. This results in continued and prolonged stimulation of antibody production by the body. It is suggested that the lipoids neutralize the toxicity of the vaccine to some extent. The advantage of being able to give all the vaccine in one dose, especially in an emergency such as in the mobilization of our draft army is obvious. The disadvantages are the technical difficulties in manufacture especially in relation to obtaining a sterile product. The injection is also somewhat troublesome as a large caliber needle must be employed. The army suspended the use of lipovaccine at the close of the war.

**Testing the Potency of Bacterial Vaccines.**—Two methods are available, first the determination of the degree of protection developed by vaccinated animals, second, the determination of the antigenic value, that is, a measurement of the antibody response following injection of man or animals. While it is not established that such tests are necessarily a criterion of the actual immunizing value in man, the insistence upon such tests by the controlling authorities (Hygienic Laboratory) is of great importance. Such tests will increase the care in production and will prevent sophistication, intentional or unintentional, through the use of cultures not true to name. An antigenic standard has been placed on typhoid vaccine, the agglutinin response being used for expediency sake.[1] Similar standards will undoubtedly be placed upon other vaccines in the near future. In some instances it will probably be necessary to employ the protection method (pneumococcus) because the irregular and low antibody response.

*Length of Potency of Vaccines.*—No definite knowledge is available. Until this is available it would seem advisable to limit the period to possibly six months. Lipovaccines, theoretically, should retain their antigenic properties indefinitely, because of the dried condition of the bacteria.

**Therapeutic Application.**—*Theoretical Conditions.*—The use of bacterial vaccines in the course of an infection was primarily based on the conception that the infection was not a sufficient stimulus to the production of adequate curative antibodies. The additional stimulus of vaccines therefore would increase the antibody production and hasten cure. This conception is based on the idea of specific action. On this basis, localized lesions of a subacute or chronic character should be the type of case most benefited. Although this is, in general, the fact, the more acute and general types of infection are also benefited by vaccine therapy. It would seem that when the blood stream was invaded by bacteria, all the tissues of the body would be stimulated to their maximum capacity of response. We must, however, distinguish three types of generalized invasion: first, where there is an initial blood invasion with secondary localization and the disappearance of the infective agent from the blood; second, acute local infections, with limited invasion, without multiplication in the blood (simple bacteremia); third, a septicemia, invasion and multiplication in the blood stream.

The first two show the ability of the body to limit the blood infection by a response of the protective forces of the body. This, however, may not be the maximum possible response and in these conditions vaccines do increase the antibody response, an example being typhoid fever. Furthermore, the injection of vaccines leads to a cellular response in the form of a polynuclear leukocytosis, or an increase if already existent.

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[1] McCoy: Am. Jour. Public Health, 1918, viii, 299.

It is evident, however, that injudicious dosage might overwhelm the body and break down the ability to respond, with serious consequences to the patient. In the septicemia types, intravascular multiplication with no tendency to localization usually indicates the inability of the body adequately to respond, and little is to be expected from the further stimulus of vaccines. This is usually found to be the case.

Considering specific response to vaccines, one would not expect much benefit in acute self-limiting types of infection, as the antibody response requires several days to become marked.

Theoretically, if the antibodies cannot reach the focus of infection in sufficient concentration, vaccines would be of little help. This is probably a factor in the generally poor results obtained with infections of bone sinuses or cavities.

Vaccines are an aid, not a substitute, for indicated surgical procedures, and the application of the latter should never be delayed. Incision, drainage and relief of tension, even if pus has not formed, are of curative value due partly to better circulation, also to the fact that drainage allows the exudation of fresh serum and cells, both of the utmost importance.

**Non-specific Response to Specific and Non-specific Vaccines.**[1]—The occurrence of prompt curative response following the injection of not only specific but also of non-specific substances cannot be explained on the basis of specific antibody response. Such a response, especially following intravenous injection of non-specific substances would at first glance overturn all our ideas of the specific action of therapeutic vaccines. The recent methods of treating typhoid fever by intravenous inoculation is a good example. The injection of an appropriate amount of typhoid vaccine or even paratyphoid or colon vaccine or albumose, is followed by a chill and a rapid rise of temperature followed by a progressive fall, sweating, and marked subjective and general improvement. At first there is a decrease, then a sharp increase of the polynuclear leukocytes. In some cases the reaction develops into a recovery by crisis, in others the disease resumes its course and a second injection, giving a similar reaction, may or may not be followed by critical recovery. Similar curative reactions have been obtained in other infections. (See Gonococcus.)

As far as we know at present, the following factors enter into such curative reactions. The sharp leukocytic response is undoubtedly of value. Increased antibody production cannot occur even with specific vaccines; furthermore, vaccination during the incubation period of typhoid fever has little, if any, effect on the subsequent disease, which should be the case if the mere increase of antibodies were the important factor. On the other hand, the best results are obtained after the tenth day of the disease, that is, after response to infection has been fully established and the injection at this time is followed by a rapid increase of the antibodies in the blood. What apparently takes place is a stimulation of the hemopoietic organs (the probable source of antibodies) with the release and dispersion of antibodies already formed and the throwing out of polynuclear leukocytes. A further factor as pointed out by Jobling is the mobilization of non-specific ferments, the serum-protease, for instance, would act on the toxic products of the bacilli and reduce them to non-toxic products. The antiferment content of the serum is also influenced and may be a factor. Desensitization or a refractory condition of the cells to typhoid bacillus products has been suggested in explanation.

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[1] See Hektoen: Jour. Am. Med. Assn., 1916, lxvi, 1591. Jobling: Archiv. Int. Med., 1917, xix, 1042. Snyder: Ibid., 1918, xxii, 224. Coure and Calhoun: Ibid., 1919, xxiii, 69. Herrmann: Jour. Inf. Dis., 1918, xxiii, 457. (Further bibliography in these articles.)

The chill and rise of temperature are important, as without these curative effects are not obtained. How much of the reaction is due to the injected protein alone, or to the dissolution products resulting from the sudden response of the body is not clear. Normal persons react similarly but larger doses are required and the reaction is less intense in the milder infections. Where there is a focal infection, reactions at the focus are undoubtedly of curative value. This is most evident in gonorrheal arthritis.

The ability to respond to non-specific or to specific substances run parallel, so that the data given as to selection of case apply equally.

Although non-specific substances are of curative value, the relative value of these as contrasted with specific vaccines, or to what degree the curative response to specific vaccines is of non-specific character, is still to be determined. Theoretically, the added specific response to vaccine of the autogenous type should be of value. Non-specific substances may raise the resistance to specific infection, but for prophylactic purposes specific vaccines must be employed.

The knowledge we have is no excuse for ignoring specificity nor for the general use of mixed and pseudospecific stock vaccines. It is unfortunate that many reports of beneficial results of vaccine therapy are valueless in this connection as the results are based on the assumption of specific action whereas the data given are no guarantee that such was the case and deductions are therefore impossible.

**Reaction to Vaccines.**—The reaction may be local, focal or general. As a rule, with appropriate dosage reactions should be slight or, at most, moderate. With intravenous therapy a reaction (see above) is essential.

**Dosage.**—The dosage given for prophylactic purposes is based on experience and should be closely adhered to. Children stand vaccines well, and full correction according to weight is unnecessary. For therapeutic purposes it is usually better to start with small doses and increase these until the maximum is reached, which is usually the reacting dose in the individual. The period between injections may be from twenty-four to seventy-two hours or longer. Continued injections increasing in amount frequently lead to cure even when no beneficial results are seen at first.

**Control of Dosage**—The use of the opsonic index (see p. 223) has been abandoned, the dosage being based on reaction and clinical results.

**Negative Phase**—Wright thought that there was an initial perio depression following vaccination. Unless the dose is large or reckl administered, this need not be considered. Prophylactic vaccin can be carried out even though the person is exposed to infection a not injurious even during the period of incubation. At most it accentuate the onset of the disease.

**Mode of Injection.**—This is usually subcutaneous. The intrave method should not be undertaken unless one is conversant with method and has a thorough understanding of its possible dan Intravascular agglutination with cerebral embolism, shock due to r dissolution of bacterial products and hemorrhage as in typhoid feve the possible dangers. Several deaths can be directly attributed to mode of injection. Intramuscular injections may also be used.

## PRACTICAL APPLICATION OF INDIVIDUAL VACCINES.

The following is a brief summary of the modes of administration the results in various types of infections.

**Staphylococcus Infections.**—The best results have been obtaine chronic or recurrent types of infection, such as acne and furuncul Stock vaccines may be employed, but if failure is encountered autoge vaccines should be tried. In the deep indurated types of acne o bacteria are probably important, as B. acne, and vaccines of the bac in doses of 2 to 20 million should be tried with the staphylococcus. furunculosis the vaccine seems to be of more value in preventing lesions than in the cure of the existing foci. Sycosis and other lesions with associated pustular lesions may be benefited by vacci Acute local lesions are probably little influenced, and in this and in other conditions mentioned the usual modes of treatment should employed. The greatest care should be taken to protect the heal skin from discharges from the infected focus.

**Dosage**—The more extensive the lesions the smaller should be initial dose. From 100 to 1000 million is the average increase in dos though larger doses may be given.

**Streptococcus Infections.**—**Local Infection.**—Acute infections due Streptococcus pyogenes are usually surgical conditions. In the subac stage vaccines may be of help. Streptococci are found in many c ditions, such as common colds, bronchitis, sinus involvement and mo infection, but vaccines are of doubtful value. Puerperal infections probably little affected by vaccines. The reported results in erysip are as variable as the disease itself. Erdman in an analysis of 800 ca could see no result.[1]

**General Infections.**—The data are insufficient. There is a slight indi tion that immune serum followed by vaccines is of some value.

**Dosage.**—In severe or general infections an initial dose of 5 to million, in more local lesions larger doses, may be given. The maxim is usually about 500 million.

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[1] Jour. Am. Med. Assn., 1913, lxi, 2048.

**Pneumococcus Infections.—Pneumonia.**—Only a moderate number of cases have been treated under conditions of specific relationship of vaccine to the infecting pneumococcus. (See types under Pneumococcus.) Favorable reports have been made without regard to this relationship. It may be that this disease can be favorably influenced by non-specific therapy; Rosenow[1] believes that very good results, clinical improvement, shortening of the disease and lowering of the death-rate is brought about by the injection of partially autolyzed pneumococci. The earlier the administration the better were the results. His results cannot be analyzed in relation to whether the results recorded are due to specific or non-specific action. The temperature charts presented indicate very strongly that the results were due to non-specific protein action. Type determination is not given although he advises a "polyvalent!" antigen, presumably he means one containing the three fixed types. It would be of interest to repeat his work and determine whether pneumonia due to Group IV reacted the same or differently from those due to the fixed types.

**Dosage.**—Ten to 500 millions.

**Prophylactic Vaccination.**—Wright's work anticipated our present knowledge of the types of pneumococci. Lister determined first the dominant types in the area he was working in (South Africa) and prepared his vaccine accordingly. During a period of nine months following the vaccination not one of 11,000 vaccinated developed a pneumonia due to pneumococci of the types contained in the vaccine. Eighty-two cases due to other types did, however, develop. Cecil and Austin[2] obtained similar results with a vaccine containing Types I, II and III. They vaccinated over 12,000 men. Their results are difficult of interpretation as they obtained not only a decrease in pneumonias due to the fixed types but a proportionate decrease in pneumonia due to Group IV, Streptococcus viridans and Streptococcus hemolyticus. Cecil and Vaughan[3] in a later experiment used the lipovaccine. The reduction in pneumonia was again marked among the vaccinated including Group IV infections. This apparent non-specific protection has no experimental parallel and we do not believe that one is justified in believing that the fixed types will give cross-protection in man when all animal experimentation is negative. The results as they stand are suggestive but by no mean conclusive.

**Dosage.**—Saline vaccines, 6 to 9 billions of each type, divided into 3 to 4 doses five to seven days apart. Type III has been employed in relatively smaller doses than the other two types. Lipovaccines, see p. 641 contains approximately 30 billions.

**Other Infections.**—See common colds, bronchitis, otitis, etc., p. 653.

**Dosage.**—Ten to 500 million.

**Gonococcus Infections.**—Urethritis is uninfluenced and the number of complications are not appreciably reduced. Vaginitis shows very little

[1] Jour. Am. Med. Assn., 1918, lxx, 759.
[2] Jour. Exp. Med., 1918, xxviii, 19.
[3] Ibid., 1919, xxix, 457.

improvement, though this may be due, as Pearce points out, to immunological differences in the gonococci from those found in adult infections.

**Epididymitis and Prostatitis.**—Some cases have been benefited. *Pelvic infection* or *general infections* are little influenced. *Periarthritis* and similar conditions have been very successfully treated.

**Dosage.**—From 25 to 500 million is the usual dosage, though up to 1 billion have been given. The dose should be rather rapidly increased until some degree of reaction is elicited. Although a reaction of any extent is to be avoided, a mild focal or febrile reaction is not only of value in indicating the limits of dosage but is also of curative value.

**Intravenous Administration.**—Bruck and Sommer[1] have advocated this method, and unusually good results are claimed not only in gonorrheal arthritis but also in epididymitis, prostatitis and even in urethritis. They used a preparation "Arthigon," 1 c.c. of which contains about 80 million gonococci. Müller and Weiss,[2] and Miller and Lusk have had good results with non-specific substances.

**Meningococcus Infections.—Prophylaxis.**—Three injections at weekly intervals of 250, 500 million and 1 billion respectively give rise to considerable antibody production. The data as to the protective value are too limited to draw any conclusions.

**Therapeutic Application.**—In some cases where lumbar puncture and serum administration, although repeated frequently, has had little effect an autogenous vaccine may be of value. DuBois and Neal recommend an initial dose of 100 to 250 million, increased to 1 billion, giving the injections every two or three days.

**Micrococcus Catarrhalis.**—See under Common Colds, etc. Dose 10 to 500 million.

**Typhoid Fever.—Prophylaxis.**—This method had its inception in the demonstration by Pfeiffer and Kolle and by Wright in 1896, that the injection of killed bacilli caused the production of the same antibodies as found in the blood of convalescent typhoid cases. In 1898 Wright inoculated 4000 men in India and Leishman supervised the inoculation of the British troops in the Boer War. In 1909 vaccination was started in the United States Army under the direction of Russell.

**Saline Vaccine.**—Various strains are used by the English, French and American Army medical men. The vaccine as advocated by the United States Army officers is prepared from a strain (Rawlings) of known antigenic value and the saline vaccine is only heated to 53° C., relying on the added tricresol to kill any bacilli not killed by this degree of heating. Unfortunately, some of the vaccines marketed are relatively inactive, possibly due to preparation, heating, preservation, or too long a labelled period of potency or a combination of these factors. Sensitized vaccines have been strongly advocated by Besredka, Gay and others. Lipovaccines are discussed below under triple vaccines.

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[1] Bruck and Sommers: München. med. Wchnschr., 1913, lx, 1185. Menser: Med. Klin., 1913, ix, 1332. Fruhwald: Med. Klinik, 1913, ix, 1799. Kyle and Mucha: Wien. klin. Wchnschr., 1913, xxvi, 1755. Bordack: München. med. Wchnschr., 1913, lx, 2622. Kreibick: Wien. klin. Wchnschr., 1913, xxvi, 2024.

[2] Müller and Weiss: Wien. klin. Wchnschr., 1916, xxix, 249. Miller and Lusk: Jour. Am. Med. Assn., 1916, lxvi, 1756.

**Administration.**—A strong degree of immunity is only conferred by two large or three moderate doses. The usual dosage is 500 million, 1 billion and 10 billion bacilli. Successive Saturdays are most convenient and it is preferable to give the vaccine in the afternoon so that the reaction, if it occurs, will occur while the subject is abed. Injection should be subcutaneous at the insertion of the deltoid.

**Reaction.**—Usually only a local tender reddened area develops. In some cases it is more extensive and there may be some tenderness of the axillary nodes. Slight constitutional symptoms may develop but a severe general reaction is exceptional. The reaction is of no importance except for the discomfort and has no relation to the subsequent immunity. There is no reason why vaccination should not be done during exposure to infection.

**Results of Immunization.**—Among the many millions of men of the British and American Army vaccinated before or during the present war with the saline vaccine there has been almost no typhoid fever. An excessive dose of infectious material may break down the protection which is only relative, but any extensive failure should raise a strong presumption that the vaccine employed was not satisfactory. (Lipovaccine, see below).

**Duration of Immunity.**—The degree of immunity decreases after two and a half years but even after four or five years the rate among the vaccinated may be only one-fourth that of the unvaccinated. Under conditions of constant exposure to infection associated with strain and privation as in the present war, the immunization should be repeated each year.

**Therapeutic Use.**—*Subcutaneous Infection.*—Watters[1] has collected and analyzed 1120 cases. Seventy-one deaths occurred, that is, a mortality of 6.3 per cent. Probably 17 cases with 15 deaths could be omitted on the ground that they were moribund when treated or for other reasons. This would give a mortality of 5 per cent. The incidence of relapse was 6 per cent. in the cases where stated. Various-sized doses were employed by the different observers, and there is no correlation between dosage and mortality or relapse incidence. Many factors enter into the death-rate of this disease and the lowered rate cannot be directly attributed to the vaccines. In the different series also, the rate varied very widely. In general, the patients treated seemed brighter and the temperature averaged lower, and the febrile period was appreciably shortened. The more moderate dosage, 250 to 500 million would seem advisable.

*Intravenous Use.*—This method was introduced by Ickikawa in 1912.[2] Several hundred cases have been treated up to date by various observers. The reaction has been described (p. 386). About 50 per cent. quickly convalesce under this treatment. There is insufficient data to warrant deductions as to its influence on the death-rate. A number of cases have developed fatal hemorrhage from the bowel or elsewhere after inoculation and several cases have died shortly after injection; in 2 there was an associated pneumonia. Evidence of hemorrhage, pneumonia or cardiac disturbance are therefore contra-indications. The dosage and vaccines employed have differed widely. From 50 to 250 million is the average. Although Gay advocates the use of sensitized vaccines, no one vaccine seems better than another. (See also under Non-specific Vaccines.)

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[1] Med. Record, 1913, lxxxiv, 518.

[2] McWilliams: Med. Record, October 16, 1915; Jour. Immunol., 1916, i, 759. Gay and Chickering: Arch. Int. Med., 1916, xvii, 303.

**Paratyphoid Infections.**—Paratyphoid fever is comparatively uncommon in this country, though under camp conditions it may became epidemic, as was the case among the militia encamped on the Mexican border. It prevailed among the European troops before the use of a vaccine containing both typhoid and paratyphoid bacilli.

**Prophylactic Vaccination.**—Vaccination is absolutely necessary among troops, etc., as evidenced by our experiences as well as the conditions in the European war. As paratyphoid is more or less endemic on the continent the occurrence of paratyphoid fever became a very serious handicap to the allied troops at the beginning of the war. The efficacy of the vaccine was quickly shown by the sharp fall when paratyphoid vaccination was undertaken. The dosage of the saline vaccine is 0.5 c.c., 1.0 and 1.0 c.c. of a vaccine containing 750 to 1000 millions of each of the "A" and of the "B" type per c.c. This may be combined with the typhoid vaccine (see next).

**Typhoid Paratyphoid or Triple Vaccine.**—Because of the inconvenience of vaccinating first with typhoid and then with paratyphoid vaccine, the two were combined. Experiments had shown (Castellani and others) that the antibody response was as good as where the vaccines were used separately, furthermore the combination did not increase the reactions to any marked degree. The first use of the triple vaccine on a large scale was in the English Army. The saline vaccine should contain 1000 million B. typhosus, 750 million B. paratyphosus "A" and 750 million B. paratyphosus "B" per cubic centimeter, the doses being 0.5 c.c., 1 c.c. and 1 c.c., seven to ten days apart. The lipovaccine content is given on p. 642. This represents probably at least 7.5 billions bacilli. The absence of reports indicates that the results in the English army (saline vaccine) have been very satisfactory. There has been considerable typhoid fever, however, in the American army. As the lipovaccine has been used to such a large extent in our army, the question naturally arises is this due to failure of the lipovaccine or due to excessive exposure? In some instances the finding of a carrier in the kitchen force or an outbreak among combat troops due apparently to the use of questionable water would seem to indicate a breaking down of protection through overexposure.[1] The wide prevalence during the offensive drives, of diarrheal conditions and also dysentery indicates that the water supplies were badly contaminated and were used without treatment or with insufficient treatment. In one instance nearly 40 per cent. of a small command became infected due apparently to the use of contaminated water, while en route to the port of embarkation (U. S. A.). Saline vaccine, however, had been used in this command as is evidenced by the number of doses of vaccine given. This marked breakdown of vaccination indicates that the use of lipovaccines was not a factor.

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[1] Public Health Reports, 1919, xxxiv, 605.

The data available is added evidence that vaccination does not confer complete protection. The sanitary control of our field troops was in many instances extremely defective especially in contrast with that prevailing among English forces. This cannot be criticised in view of the extremely urgent need of reinforcements.

Several criticisms of the lipovaccines have been made, but one is in doubt as to their evaluation because detailed statistical data is not available. One criticism is the severity of many of the reactions, the persistence of the local swellings after vaccination (non-absorption?) and possibility of leakage or actual massaging out of the vaccine from the large puncture.

On the whole we are strongly inclined to the following conclusion: The lipovaccine in its present form is primarily of value and of great value in emergencies. The dose given is probably larger than necessary but in an emergency such as our mobilization, it was better to err on the safe side. The saline vaccines are preferable in civil life and probably in military organizations where time is not a factor. Naturally further improvements[1] of the lipovaccine may necessitate a reversal of this opinion.

The duration of immunity after triple vaccine is probably several years or longer under average conditions. Under conditions of stress, the vaccination had better be repeated each year.

**Bacillary Dysentery.**—Dysentery vaccines are highly toxic and Shiga has employed the simultaneous injection of vaccine and serum. The results are not wholly satisfactory, although the mortality among the vaccinated was lowered. The prevalence of different types of bacilli adds to the difficulties. It has been claimed that the vaccines are of value in the treatment of carriers. Whitmore has prepared a lipovaccine.[2]

**Plague.**—Prophylactic vaccination gives a relatively short period of immunity and is best undertaken during epidemics. The protection is only relative against bubonic plague but the mortality is also lowered. There is less protection against pneumonic infection.[3] Haffkine advises 3 to 3.5 c.c. of his specially grown broth cultures, giving a second dose after eight to ten days. Kolle advises 2 mg. of the growth on agar. These amounts are fairly equivalent to 500 million bacilli. Hitchens at the Army Medical School has produced a lipovaccine which, however, has been only used experimentally.

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[1] Whitmore has experimented to some extent with drying the bacilli in frozen condition, hoping to limit autolysis and severity of reaction.

[2] Jour. Am. Med. Assn., 1918, lxx, 902.

[3] Strong and Teague: Philippine Jour. Sc., 1912, vii, 229.

CEREBROSPINAL FLUID.—Ten c.c. of the fluid withdrawn puncture should be centrifuged for fifteen minutes, and should be examined under 150 to 200 diameter magnification

*Cultures* sometimes give positive results, especially from in lower animals and in infections with *T. rhodesiense*.

THE INOCULATION TEST.--If the trypanosomes cannot by the above methods, animal inoculation should always Monkeys, if possible, should be used, or if monkeys cannot be dogs or rats may be used. A few drops to 1 c.c. of the tissue from the suspected animal should be inoculated or subcutaneously.

Smears may be stained by any modification of the method. Giemsa's method gives good results (Plate IV,

**Prophylaxis.**—The disease is readily controlled by ures. There should be strict quarantine regulations importation of animals. When the disease has once following general measures should be taken: (1) should be isolated. (2) All infected animals should (3) As far as possible, all biting insects should be land in the neighborhood of human habitation should tivated. (4) The bodies of infected animals should biting insects for at least twenty-four hours after death. animals should, if possible, be made immune. (6) should be used to exterminate the reservoirs. In tricts the natives may be removed from the fly area.

**Treatment.**—The whole question of treatment mental stage. The chronic course of the disease after long intervals makes it impossible, especially trypanosomiasis, to come quickly to a conclusion ciency of any drug. Many drugs have been found cidal properties to a certain extent.

Atoxyl (p-amino-phenyl-arsenic acid), introduced used first by Thomas and Breinl in treatment of proved to have a beneficial effect in the different

The good reports received from the use of duced by Ehrlich,[1] namely salvarsan and

**Serum Therapy.**—Various normal sera been tried with practically no success. Thus Laveran and Mesnil[2] state that hu quantities shows manifest action on the cure results in mice and rats. Further with arsenic they obtained still better and Blandford showed that animals infection were immune to further infecti have made a very careful study of

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[1] München. Med. Wchnschr., 1909.

[2] Trypanosomes and Trypanosomia

vaccine, the other with a
culturally, differs com-
results about as good as
we were dealing with a
condition.[1]
illion, 1 billion and 2 billion
ildren under one year receive
improvement is not marked,
wise doses up to 5 billion or
every third day, the doses
espectively.
Influenza, see below. The
of the mucous membranes,
necessarily an indication of
of mixed vaccines containing
roblematical. The use of autog-
ints strongly to their etiological
estive results.
million, which can be increased

in chapter on Tuberculosis).—
in pulmonary phthisis have been

**Disease.**—In general the conclusion
insufficient if the focus is not eradi-
are not necessary. Each case is a
of typical case reports is necessary
Streptococci and gonococci are the
isms.
*Common Cold.*—The beneficial results
should be viewed with skepticism. Little
of common colds. Some outbreaks are
ding to Kruse and Foster. Micrococcus
pneumococci, streptococci, B. segmentosus,
the predominating organisms. The types
of heterogeneous groups and it is
vaccines can have any influence at least
inoculation of such vaccines may
fluence on

*Mouth Infections.*—The use of vaccines in pyorrhea is advocated by some observers but they agree that local treatment is necessary as well, whereas others find that local treatment alone is all that is necessary. The vaccines employed only represent a small part of the aërobic flora, and the dominant anaërobic fusiform bacilli and spirochetes are ignored. Whether vaccines influence the general symptoms which may be associated is another problem. (See Focal Infections.)

*Selection of Vaccine, Dosage, etc., in above Conditions.*—An autogenous vaccine based on a careful bacteriological examination is alone applicable. The stock vaccines widely advertised are, so far as our present knowledge goes, non-specific vaccines, even though the contained organisms bear the same names as those encountered in the inflammation. The dosage for the individual types has been given. Pro rata reductions should be made according to the number of types in the vaccines.

**In Diseases of Unknown Etiology.**—**Typhus Fever.**—The growing conviction that the bacillus isolated by Plotz is not the primary etiological agent in typhus lead to skepticism of the reported[1] success of prophylactic vaccination with this bacillus, as has been noted, animal immunization with such vaccines seems impossible.

**Epidemic Influenza.**—*Prophylactic Vaccination.*—During the recent epidemic the degree of reported success is directly proportional to the degree of the reporter's failure to critically control and analyze his figures. Wherever an experiment was carried out under perfect conditions of control and analysis the results indicate complete failure not only to prevent the disease but to modify the incidence of complications and their severity.[2] As far as we can determine, the disease when introduced in any group of persons, was most severe and more frequently complicated by pneumonia in the earliest cases. The tendency then was for the disease to diminish in severity. In any group from one-sixth to one-fourth or more would become infected, the remainder apparently being immune, although sporadic cases or even a recrudescence of a considerable number of cases might develop among the latter after the main wave of the outbreak was over. The apparently brilliant results of vaccination were obtained when vaccination was started at or near the peak of the case wave. It did not seem to make much difference what vaccine was used under these circumstances. In one instance the personnel of a manufacturing firm reported to us the startling results following only one dose of a commercial mixed vaccine (so-called common cold prophylactic vaccine). An analysis of the figures showed clearly that the peak of incidence had been reached when the vaccine was given. Similar results have been observed by us in the vaccination of large military groups, giving several billions of influenza bacilli. Even where an invaccination control group was not retained for comparison, the relative frequency of disease after vaccination is added and strong evidence of the failure of the vaccine.

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[1] Plotz, Olitzky and Baehr: Jour., Am. Med. Assn., 1916, lxvii, 1597.

[2] McCoy: Jour. Am. Med. Assn., 1918, lxxi, 1997. Minaker and Irvine: Jour. Am. Med. Assn., 1919, lxxii, 847. Rosenau: Jour. Am. Med. Assn., 1919, lxxii, 31.

Vaccines of B. influenzæ isolated in this epidemic, either alone or in combination with pneumococci, streptococci, and other varieties such as staphylococcus, micrococcus catarrhalis, etc., were employed. The dosage was high in most instances totalling 5 billion or more. As most of these varieties belong to *practically* heterogeneous groups,[1] which we now know to be the case with the influenza bacillus there is little if any theoretical basis for the hope that such vaccines will prevent the disease or its complications in any considerable number of persons.

*Therapeutic Use.*—Vaccines,[2] usually of mixtures of the influenza bacillus and the various cocci, have been employed with apparent success especially in the bronchopneumonias. Some of the comparisons of the severity and mortality among vaccinated and unvaccinated are unjustified, however, as the cases compared were not comparable in point of time. Like with prophylactic vaccination the importance of comparing only simultaneous controls was lost sight of. There is some indication of beneficial results in the individual cases, evidenced by a drop in temperature and subjective improvement. As such beneficial results as were obtained came on promptly, also because the vaccine was probably not specifically related to the infecting types, as well as the similar results obtained with non-specific proteins,[3] leads one to believe that the benefits obtained were due to non-specific protein action.

[1] The recent report of Rosenau does not change our opinion in this regard.

[2] Wynn: Lancet, 1918, ii, 874. Roberts and Cary: Jour. Am. Med. Assn., 1919, lii, 922.

[3] Snyder: New York Med. Jour., 1918, cviii, 843.

# CHAPTER XLVII.

# THE PRACTICAL APPLICATIONS OF SERUM THERAPY.

THE advisability of using sera in any particular disease is influenced by a number of considerations. The primary one is whether a serum has been obtained and is available which contains antibodies of suitable kind and amount to neutralize the toxins or aid the body cells and ferments in destroying the microörganisms. Others almost equally important are whether the infections can be identified from clinical signs alone, or only after additional laboratory examinations, and whether the sera can be brought into contact with the toxin or organisms at the essential points, in the necessary concentration and within the required time. Practical serum therapeutics in the more important infections in which some results have been obtained will be considered in the light of the above considerations.

## SERUM TREATMENT OF LOBAR PNEUMONIA.

This disease is so uniformly due to one organism that from the clinical signs alone the physician can almost assume a pneumococcus infection. This knowledge is not as valuable as it seems since it has become evident that the characteristics which define a pathogenic microörganism are frequently so broad as to include a number of strains, which from the viewpoint of immune sera are as distinct as microörganisms with wide cultural difference. The term "pneumococcus" as pointed out by Neufeld is one of these broad group names which cover a number of strains each one of which is little if at all affected by the antibodies produced through immunizing injections with the others.

A further extremely important differentiation between the types is that with our present methods some excite by their injection into suitable animals abundant antibodies, while others do not. The reasons for this we do not understand. The most recent and most thorough investigation concerning the value of the specific serum for pneumonias due to each type of organisms has been carried on at the Rockefeller Institute Hospital by Cole and his associates and the present favorable outlook for treating a certain proportion of pneumonia cases is largely due to their work. (See chapter on Pneumococci.)

Cole's, Longcope's and Richardson's recent statistics of the frequency and the mortality of pneumonias due to the types are of great practical interest.

It is noticed that about 30 per cent. of the cases of pneumonia and about one-third of the total deaths are caused by infections with type I.

These figures are very important because at present the results of serum injections follow their antibody content and seem favorable in type I infections, doubtful in type II and negative in type III and in type IV. Since the influenza epidemic the type IV strains have been more abundant than the combined fixed types I, II and III.

| | Rockefeller Institute (P. H. Longcope). | | | | | | Univ. Penna. Hospital (Richardson). | | |
|---|---|---|---|---|---|---|---|---|---|
| | Number. | | Incidence, per cent. | | Mortality, per cent. | | No. | Incidence, per cent. | Mortality, per cent. |
| | Cole. | Longcope. | Cole. | Longcope. | Cole. | Longcope. | | | |
| I | 78 | 13 | 33 | 23 | 25.0 | 12.5 | 60 | 31 | 30 |
| II | 75 | 11 | 32 | 21 | 29.0 | 72.7 | 39 | 20 | 25 |
| III | 22 | 7 | 9 | 14 | 45.0 | 85.7 | 13 | 6 | 50 |
| IV | 48 | 21 | 20 | 40 | 12.5 | 23.8 | 83 | 43 | 12 |
| Other bacteria | 14 | .. | 6 | | | | | | |

**Method of Administration.**—The experimental work in animals and the observation of cases has led to the general use of larger injections and the substitution of the intravenous for the subcutaneous method. The size of the patient and the strength of the serum in antibodies have not been considered in controlling the size of the dose, although all accept the fact that theoretically they should. The serum should be standardized, as in the case of antitoxins, 0.2 c.c. of a recent serum should protect against at least 100,000 f. d. of a very virulent type I pneumococcus. As serum of this strength is easily prepared, there is little justification for the use of weaker serum.

Cole advises that 80 c.c. to 100 c.c. of serum diluted with an equal quantity of salt solution be injected intravenously and repeated about every twelve hours until permanent improvement is noted. Usually he gives three to five doses.

The repetition of the dose every twelve hours is founded on the desire to give it sufficiently often. Cole states that the transferred antibodies tend to disappear after a dose of serum. This is due, in his opinion, to combination with the antigen in the body. The degree of disappearance after repeated injections is proportional to the severity of the infection. When antibodies begin to persist after an injection, recovery usually follows. In our own experimental work we have found that a single dose of 0.1 c.c. protects a mouse for the next four days from an injection of 1000 fatal doses of living pneumococci, and as late as the fifteenth day, from 100 fatal doses. The mouse differs from the sick person in not having an infected area and associated free antigen in the blood and other body fluids. (See Pneumococcus.)

For careful comparative work the actual protective value of the serum at the time of use should be known. With serum as now produced under Federal supervision, the minimum strength allowed is that given above.

Cole recommends that the serum be only given in a case after the bacteriological test has shown the type. We believe that when in severe cases a delay in the typing is unavoidable a first dose of type I serum should be given as soon as possible and the later injections only after the bacteriological report. Since the influenza epidemic there has been a much smaller percentage of cases due to type I. Cole's plan means in most cases a delay depending upon the availability of laboratory help as well as upon the suitability of the specimen for the rapid methods of testing (see Pneumococcus). This delay may extend to twenty-four hours or longer and may prevent the use of the serum at all.

It is understood that the severe cases which receive an injection of type I serum and from which no bacteriological report is received cannot be considered as having any value in forming an opinion of the effect of the treatment;

Thus if Dr. Longcope had treated all his cases with type I serum and had not identified the infecting type in each of the 22 deaths among his 52 patients he could have had no conception of the value of the serum, for only one of the deaths and 13 of the cases were due to the type I organism.

**The Results of Large Intravenous Injections of Specific Serum in Type I Infections.**—There is in about 30 per cent. of the cases an almost immediate more or less severe chill with a considerable rise of temperature which lasts for a short period. This occurs usually after the first but sometimes after the later serum injections. If the blood contained pneumococci, they disappear within twelve hours after the injection. The temperature usually rapidly falls after the initial rise to a point lower than before the rise and the symptoms, as a rule, improve sooner than the average untreated case of equal severity. The mortality up to the present time has been much less than in the untreated cases. Serum sickness with rashes, painful joints, swelling of lymph glands and other symptoms occur to a greater or less extent in about 50 per cent. of the cases during convalescence. The serum sickness, while it lasts, is very annoying, but not dangerous. The serum has very definite preventive and therapeutic value in the experimental pneumonia of monkeys due to the fixed types.

The fact that such really promising results have been obtained in human cases due to type I leads us to hope that Wadsworth, Cole and others will succeed in their endeavors to get a therapeutically valuable serum for types II and III and possibly so concentrate the antibodies as to use a combined polyvalent serum.

## SERUM TREATMENT OF EPIDEMIC MENINGITIS.

The intraspinal method of injecting serum from horses immunized to meningococci in meningitis introduced by Jochmann has been approved by all. The collective investigation carried out under Flexner's supervision practically settled its value, and if it were not for the somewhat discouraging reports received from England during 1915 it would not be necessary to more than allude to the value of the serum.

Several reports from England mention a mortality under serum treatment of different collections of cases of from 52 to 63 per cent. and several experienced men have advised against the use of the serum. Should these results in any way cause us to change our favorable opinion?

There are undoubtedly certain epidemics in which an unusual proportion of the cases develop a thick tenacious exudate which greatly hinders the successful use of the serum. A number of these have been found at the New York State Quarantine Station. Again the sudden great foreign demand for the serum caught some of the manufacturers with a small supply and the attempt to replenish their stock caused them to bleed their horses too frequently and thus obtain a serum which was found at a later period to be poor in antibody content. Unless the strains used are properly selected the antibodies may not be suitable to combine with the strain producing the local epidemic.

That one or more of these explanations for most of the poor results are true is rendered certain by the fact that when potent serum was used in several thousand cases among the English troops in 1916 and later among our own troops results were very good. Since 1910 the New York City Health Department bacteriological laboratory has treated all cases of epidemic meningitis applying to it. Our mortality in different years has varied from 21 to 34 per cent., with an average in the last few years of about 23 per cent. Drs. Sophian, DuBois and Neal, who have had charge of the serum treatment for the laboratory, are absolutely convinced of the value of the treatment, not only from the clinical course but from the changes which take place in the spinal fluid as shown by the disappearance of the meningococci.

**Administration of the Serum.**—Dr. Neal finds that too often the private physician fails to repeat the injections with sufficient frequency. It is very rare, except in a case already convalescent, that it is correct to give daily injections for less than four days. If the organisms or symptoms do not disappear the injections of 10 to 25 c.c. of serum should be continued for many days. Fortunately lumbar puncture with removal of fluid is of value in the treatment. Unlike pneumonia, practically all cases of epidemic meningitis can apparently be treated by a single serum. The different strains do, indeed, differ, but they have group relationships and intravenous injections of suitably selected strains cause the horse to produce a serum capable of influencing almost all strains and apparently almost all cases. The fluid removed from every case of suspected epidemic meningitis should be examined microscopically and culturally, for only in this way can a correct diagnosis be made.

**Directions for Use of Serum.**—The following directions are issued by the Research Laboratory of the New York City Board of Health.

Perform a lumbar puncture under aseptic precautions in the third or fourth lumbar space. A general anesthetic should never be used. In hypersensitive patients a local anesthetic may be advisable. Have the patient lying on the side with the back arched so that there will be the greatest possible distance between the spines of the vertebræ.

Find the notch nearest a line connecting the crests of the ilia. Introduce the needle, preferably a Quinke needle, in the midline and push forward and a little upward. The distance the needle goes in depends upon the age of the patient and the muscular development. It varies from ½ to 3 inches. Allow the cerebrospinal fluid to flow out until the pressure is so reduced that only 3 or 4 drops come per minute. If the fluid is cloudy, inject the serum immediately.

The serum is warmed to body temperature and injected very slowly under the least possible pressure. A funnel and the tube arrangement allowing it to run in by gravity should be used. The barrel of an ordinary syringe may be used as a funnel. The rubber tubing should be ⅛ to ¼ of an inch in diameter and long enough so that the funnel can be raised 12 to 15 inches. In general, the average dose for an adult is 20 to 40 c.c. and for infants and children up to 20 c.c. The amount depends as much upon the quantity of cerebrospinal fluid withdrawn as upon the age. An infant will frequently stand 10 to 20 c.c. without difficulty. The dose should usually be at least 5 to 10 c.c. less than the amount of cerebrospinal fluid withdrawn. When serum apparently runs in freely after a dry tap, it is advisable to proceed very slowly and to watch the patient carefully for the slightest change in pulse and respiration. In cases with very thick exudate which will not flow through the needle, gentle suction with a syringe may be tried. If that fails, a little serum injected will sometimes start the flow. When possible, further injections are made only after bacteriological examination has determined the cause to be the meningococcus. The antimeningitis serum does no harm in meningitis due to other organisms.

In certain severe cases it may be advisable to inject the serum every twelve to eighteen hours until there is improvement. In moderate and mild cases it should be repeated each day for the first four days. Further administration depends upon the patient's general condition and the bacteriological examination of the fluid. Usually four to six injections are necessary, but as many as fifteen or more may have to be employed.

During or immediately after the injection of serum the respiration may entirely cease or the pulse may become very rapid and thready. Such an occurrence, while alarming, is not necessarily serious and is best treated by immediate withdrawal of some of the serum if the needle is still in place. If the needle has been withdrawn, or, if after some of the serum is removed the symptoms do not ameliorate, artificial respiration should be resorted to for the respiratory condition and adrenalin or other stimulants hypodermically for the heart.

Of late considerable attention has been drawn to the intravenous use of serum through the work of Herrick at Camp Jackson. While this method is undoubtedly of value in meningococcus septicemia with or without meningitis, its usefulness and limitations should be kept in mind. The intravenous administration of serum cannot be relied upon to influence the meningeal condition as only a very small percentage of the antibodies of the serum will pass to the spinal fluid. On the contrary the serum injected intraspinously gradually passes into the blood stream and will therefore influence the septicemia. We believe that the intravenous injection should be limited to those cases in which the septicemia dominates the clinical picture. These cases have largely been confined to the men in large military camps.

The successful treatment of cases of meningitis must always depend upon experience and judgment. It cannot be reduced to a rule of thumb.

In all cases with meningeal symptoms a lumbar puncture should be done. No ill effects follow, on the contrary the relief of the pressure frequently produces beneficial results.

## ANTISTREPTOCOCCIC SERA.

The same reasoning applies as with the antipneumococcic serum and the same dosage and method of administration.

Some of the strains recur frequently, others infrequently in infections. If we suspect that the infection is due to the hemolytic type of organism we can rightly give this type of serum with the hope of some good resulting if the serum matches the infecting type.

In the viridans type of infection we have no evidence that any good is done by serum. This is probably due to the fact that each organism has different antigenic properties. In this characteristic they form a practically heterogeneous group.

The organism should be identified as soon as possible, if sufficiently virulent in mice it may be tested against the serum in a mouse, to determine whether the serum available has any protective antibodies for the infecting strain. Much further combined clinical and laboratory investigation is required before a decision can be reached as to the value of the serum and the best dosage.

If it were not for serum sickness there would be no question that injections should be made early before the infection has advanced and the streptococci acquired a somewhat greater resistance to the specific effect of the serum antibodies and ferments.

The repeated local bathing of infected tissues with the antistreptococcic serum seems to have a more beneficial action than that exercised by a non-specific serum.

## THE SERUM TREATMENT OF BACILLARY DYSENTERY.

The earlier opinion has been confirmed that bacillary dysentery alone occurs in cold and temperate climates while in hot climates both bacillary and amebic cases occur in about equal numbers. The idea that summer diarrheas were frequently due to the dysentery bacilli has been discarded. The bacilli may be present at times in small numbers in these cases, but they have too little part in the disease process to require specific treatment. The dysentery bacilli may be divided into dysentery bacilli and paradysentery bacilli or into different strains of a group. Like so many other bacteria causing inflammations of mucous membranes the organisms exciting dysentery belong to a variety of strains.

For sporadic cases and those occurring in the beginning of an outbreak, a polyvalent serum must be used. In later cases in an epidemic where the strain has been identified the monovalent serum is to be used if possible.

The serum in mild cases is given in doses of from 10 to 30 c.c. twice daily according to the size of the person. In severe cases as high as 100 c.c. two or three times in the twenty-four hours can be given and in desperate cases the serum has been given intravenously. If given intravenously, the serum must be warmed and given slowly. The doses are to be continued from day to day until permanent improvement is established. Injections are usually continued for two or three days. The majority of those who have used the serum confirm Shiga's original belief that the results are good. Our own experience in a number of severe cases is on the whole favorable. We believe it is unnecessary to use the serum in slight cases. In favorable cases within six to twelve hours the constitutional symptoms frequently improve. The abdominal pains are less, the mental condition is better and the pulse slower and stronger. There is frequently at this time some reduction in the number of stools, though they may be more copious and have increased sloughs. The serum on the market is not standardized and some of it is valueless. The necessity of using a polyvalent serum in most cases also lessens its value. In spite of these objections the use of the serum in severe cases is strongly indicated. It is very desirable that records be kept of the cases treated and the results reported.

Shiga believes the mortality to be reduced by the serum treatment from around 35 per cent. to about 9 per cent. Some report even more striking results, while still others have met with disappointment. Undoubtedly the extent of mixed infections with other organisms, the possibility of some cases having been due to amebas and the use of inactive serum must be considered as being possibly the cause of the failure of the serum in these cases. Ruffer and Willmore showed that the Shiga type of serum had no effect on their cases which were due to P or Y type of paradysentery bacillus. Either a polyvalent serum active against the prevailing types, or a suitable monovalent serum was found effective. The usual serum after-effects may develop.

## THE THERAPEUTIC USE OF HUMAN CONVALESCENT BLOOD HAVING SPECIFIC ANTIBODIES.

Convalescent serum, plasma or whole convalescent blood has been used in the treatment of early toxic cases of scarlet fever both here and abroad and has given encouraging results in the limited number of cases observed thus far. Reiss and Jungman recommended the intravenous injection of 50 to 100 c.c. of pooled convalescent serum, while Zingher makes use of the intramuscular injection of whole convalescent blood, citrated or non-citrated, which he injects in quantities of 120 to 240 c.c. The blood causes no local inflammatory reactions in the muscles, and is rapidly absorbed. The convalescent serum or fresh whole blood is obtained from patients who are two or three weeks convalescent from scarlet fever. These donors should be free from syphilis and tuberculosis.

The effect of convalescent serum or whole blood in the uncomplicated early toxic cases of scarlet fever is seen in a critical drop in temperature, beginning about six hours after the injection and ending in from twenty-four to thirty-six hours; an early fading of the rash; improvement in the circulation and character of the pulse; and especially in the general condition and mental symptoms of the patient. Zingher reported the results obtained at the Willard Parker Hospital with intramuscular injections of whole convalescent blood in 15 very toxic cases of scarlet fever, selected out of a total of 900 admissions. A striking improvement was noted in 5 cases, improvement and final recovery in 6 more, while 4 patients died from various septic complications.

In the later septic cases, seen from the fifth to tenth day of disease, complicated by an extensive streptococcus exudate over fauces and tonsils, enlarged and tender cervical glands, a poor circulation and showing general septic temperature, fresh normal blood, injected in quantities of 120 to 240 c.c. and repeated if necessary in three or four days has shown very beneficial effects in some desperately ill cases. Fresh normal blood has no specific action in septic cases of scarlet fever, but it supplies definite nutritive, stimulating, and normal bactericidal substances.

A few investigators[1] have reported successful results in preventing measles by convalescent human serum, either alone or mixed, with measles "virus."

In the pneumonia[2] following influenza considerable effect seems to have been obtained by the use of convalescent blood or serum. As with vaccines direct comparison of treated and untreated controls simultaneously observed are not available so that the conclusions are primarily based on the apparent clinical effect. In the series treated by McGuire and Redden over half reached a normal temperature within twenty-four hours of the injection. Such a result is very strongly suggestive. The results were apparently better the earlier the serum was administered.

## USE OF DIPHTHERIA ANTITOXIN IN TREATMENT AND IMMUNIZATION.

The antitoxin in the higher grades of globulin solution or serum is identical with that in the lower grades; there is simply more of it in each drop. In treatment, however, for the same amount of antitoxin we have to inject less foreign proteins with the higher grades, and therefore have somewhat less danger of chills, rashes and other deleterious results. The amount of antitoxin required for immunization is 300 to

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[1] Richardson and Connor: Jour. Am. Med. Assn., 1919, lxxii, 1046.

[2] McGuire and Redden: Jour. Am. Med. Assn., 1919, lxxii, 709. Maclachlan and Fetter: Ibid., 1918, lxxi, 2053. Ross and Hund: Ibid., 1919, lxxii, 640.

500 units for an infant, 500 to 1000 for an adult, and proportionately for those between these extremes. The larger doses are advised when the danger of infection is very great. After the observation of the use of antitoxin in the immunization of many thousand cases, we have absolute belief in its power to prevent an outbreak of diphtheria, when given in the amounts advised, for at least twelve days and also of its almost complete harmlessness in the small doses required. When double the above quantities are given the immunity is prolonged, on the average, for about one week. If it is desired to prolong the immunity the antitoxin injection is repeated every ten days.

**Treatment.**—Although more than twenty-five years have elapsed since the introduction of diphtheria antitoxin in the treatment of diphtheria, good observers, although nearer together than at first, still differ in the amount which they believe should be injected and in the method of its administration. Before giving our own conclusions on the proper dosage, it is well to consider several important points upon which this dosage is founded.

The amount of toxin in any case of diphtheria is comparatively small. One hundred units of antitoxin which would neutralize fifty times the amount of toxin sufficient to kill a six-year-old child, would surely make harmless all the toxin present in the most malignant cases if it could gain access to it in time. If we gave antitoxin, therefore, as many suppose, simply in sufficient amount to neutralize the poison in the body of an infected person, comparatively small amounts would be injected, but we have to give very much more than this because of the time it requires for much of the antitoxin to reach the toxin. This can be brought into direct contact with the toxin only by being absorbed into the blood and then passing through the capillary walls to the tissue fluids and cells. The greater the quantity of antitoxin that is in the blood, the greater will be the speed that an appreciable amount will pass to the tissues. The combined endeavor of the clinical observer and the laboratory worker is to find the suitable dose which will give a sufficient concentration in the blood to neutralize, as quickly as necessary, the toxin in the tissues. In the laboratory we can test the amount of antitoxin which is absorbed into the blood from any given dose and the amount which passes out to the tissues, while the clinical observer can note the changes which take place as he watches the case after antitoxin treatment.

It is naturally a matter of great importance as to how the antitoxin is administered. When given subcutaneously, the swelling caused by its injection rapidly disappears by the absorption of the water, but the globulins and antitoxin remain behind in the tissues because of the slow absorption of proteins. By testing many patients, it has been found that it takes twenty-four hours for the major part of the antitoxin to be absorbed by the blood from the subcutaneous tissues and some twelve hours from the muscles. For its total absorption it requires two or three days. Through the use of the Schick test, it has been determined that an injection of antitoxin given intravenously passes out to the tissue fluids about ten times as rapidly as when the dose is given subcutaneously, and four times as when given intramuscularly. A unit gives most effect when given intravenously and least when given subcutaneously. If it were not for the fact that it is more difficult to give it intravenously and also that sharper serum reactions occur, the intravenous method would be the only one used. Another matter which is of importance is the size of the individual treated. It is self-evident that if a child weighing twenty pounds is injected with 10,000 units, it would, on the average, have in its blood five times as much antitoxin per cubic centimeter as a person receiving the same amount who weighs 100 pounds. The influence of weight on the dose

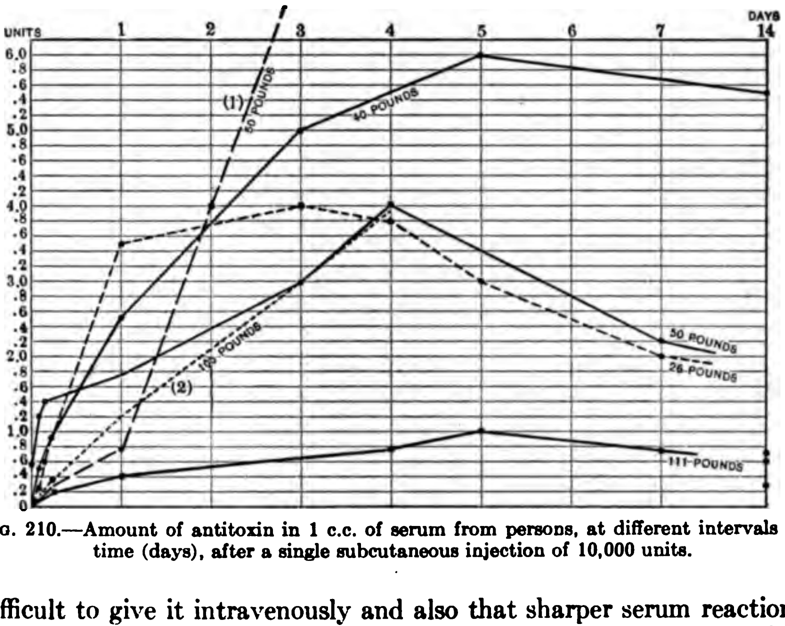

FIG. 210.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time (days), after a single subcutaneous injection of 10,000 units.

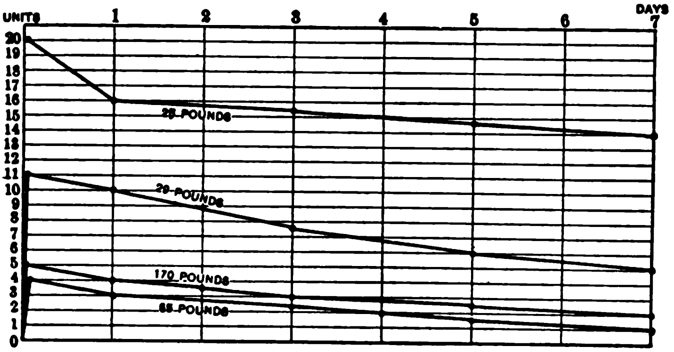

FIG. 211.—Amount of antitoxin in 1 c.c. of serum from persons, at different intervals of time, after a single *intravenous* injection of 10,000 units.

is, however, largely neutralized by the fact that diphtheria in the child is generally more dangerous than in the adult. Every minute of delay in the neutralization of the toxin in a severe case is of importance, but in a mild case, where dangerous poisoning is still remote, slight delay makes little difference. Infants and children are especially liable to laryngeal diphtheria, so that every case in a child presents a certain gravity which the adult does not present.

The last point to be considered is whether a single or a multiple dose should be given. It must be realized that antitoxin has no effect whatever on injury which has already taken place. It is as useless then as water on the ashes of a burned-out building. If the toxin is permanently united with the cell substance, antitoxin is no longer of any service. It is the early and sufficient dose which is important. When we give a divided dose, we simply get the effect of the first portion during the interval before the giving of the second dose. If the second dose had been given with the first, we would have had its effect added. and so an insufficient dose made adequate. When the first dose has been of a sufficient size, the second and third injections, though harmless, are absolutely useless. The holding back of a part of the first dose so as to give it later, simply delays its action to a time when it cannot have much, if any, effect.

For the last three years, we have used in the hospitals for contagious diseases only a single dose of antitoxin, which in mild cases, has been given subcutaneously; in moderate cases subcutaneously or intramuscularly; and in severe cases, intravenously or intravenously and intramuscularly. After twenty years of experience in treatment and animal experimentation and consultation with physicians in New York and elsewhere, the following dosage, which is that adopted by the Health Departments of the City and State of New York, is advised.

### DOSAGE OF UNITS OF ANTITOXIN IN DIPHTHERIA.

#### SINGLE DOSE ONLY.

| Mild. | Moderate. | Severe. | Malignant. |
|---|---|---|---|
| | Infant, 10 to 30 pounds (under two years) | | |
| 2000 | 3,000 | 5,000 | |
| 3000 | 5,000 | 10,000 | 10,000 |
| | Child, 30 to 90 pounds (under fifteen years) | | |
| 3000 | 4,000 | 10,000 | 10,000 |
| 4000 | 10,000 | 15,000 | 20,000 |
| | Adults, 90 pounds and over | | |
| 3000 | 5,000 | 10,000 | 15,000 |
| 5000 | 10,000 | 20,000 | 40,000 |

#### METHOD OF ADMINISTRATION.

| | | | |
|---|---|---|---|
| Subcutaneous<br>or<br>intramuscular | Intramuscular<br>or<br>subcutaneous | Intramuscular<br>or<br>½ intravenous<br>and<br>½ intramuscular<br>or<br>subcutaneous | ½ intravenous<br>and<br>½ intramuscular<br>or<br>subcutaneous |

The above amounts are sufficiently large, and I think no appreciable advantage would be obtained by increasing them. Very much smaller doses are still able to do great good, as the general blood current soon becomes antitoxic and blocks any further passage of toxin from the diseased tissues to other portions of the body. The feebly antitoxic plasma gradually permeates the body. There will be, however, more delay in the improvement of the local process, for the neutralization of the concentrated toxin in the diseased tissues will be slower. The exudate or pseudomembrane will continue to increase for some hours after the complete neutralization of the toxin because the injury to the tissue takes time to manifest itself.

**Determination of the Presence of Antitoxin in the Living Body and Results of Toxin-antitoxin Immunization.**—See p. 353.

**Danger in Giving Injections of Antitoxin.**—About 1 in 10,000 persons develop, within a few minutes after an injection of serum, alarming symptoms. About 1 in 50,000 of the injected die. About 30 deaths in all have been reported. In 140,000 persons injected by New York City Health Department Inspectors there have been 2 deaths due to serum. About the same proportion is reported from Boston. The persons suffering severe symptoms have usually been subject to asthma, while the fatal cases usually have the pathological changes known as status lymphaticus. A few of these rare cases die almost instantly. As a rule, when death occurs it takes place within a few minutes after the development of symptoms. Usually the respiratory rather than the circulatory center seems to be affected. Persons who have not reacted badly to a first injection do not need to fear a second. So far as known all fatal results have followed the first injection.

**Results from the Use of Antitoxic Globulin Solution.**—The curative effect proved to be identical with that of the whole serum. Our tests showed clearly that not only the toxin, but also the poisons produced in the animal by injections with virulent bacilli are neutralized as completely by the globulin solution as by the antitoxic serum from which it is separated. The injections of the globulin solution were found to be followed by decidedly less severe rashes than the whole serum, and it was especially noted that there were very few who had any constitutional disturbances even when the development of the rashes did occur.

The comparative table on page 668 gives a summary of the constitutional and local reactions obtained in the treatment of 50 cases of diphtheria in young children, with a lot of antitoxic serum received from three horses which were found to produce an excessive amount of disturbance, and of an equal number of similar cases treated with a solution of the antitoxic globulins derived from a portion of the same lot of serum.

The concentration of antitoxin made possible by the elimination of the non-antitoxic substances is not only a convenience but is of a distinct importance, as it tends to encourage large doses. Some producers, however, supply a product which is so rich in protein as to be almost semisolid. This is not quite so well absorbed as the less concentrated product. The total solids in the globulin solution should not be more than twice those in the serum.

The antitoxic globulin solution tends to become slightly cloudy when kept at moderate or high temperatures. This does not interfere with its potency. Substances such as solutions of carbolic acid and tricresol precipitate it but not in the quantity usually employed as preservatives.

| | Children treated with the whole serum. Per cent. | Children who were treated with the antitoxin globulins. Per cent. |
|---|---|---|
| Marked constitutional symptoms accompanied by a severe and persistent rash . . . . . . | 28 | 0 |
| Moderate constitutional symptoms accompanied by a well-developed erythema or urticaria . . | 18 | 4 |
| Very slight constitutional disturbance accompanied by a more or less general urticaria or erythema | 20 | 8 |
| No appreciable constitutional disturbance but more or less general urticaria or erythema . . . . | 4 | 34 |
| No appreciable deleterious after-effects whatever . | 30 | 54 |

Duration of Rashes.

| Days . . . . . . | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Totals. |
|---|---|---|---|---|---|---|---|---|---|
| Antitoxic globulin cases | 5 | 7 | 5 | 2 | 3 | .. | 1 | .. | 23 |
| Whole serum cases . . | 1 | 4 | 10 | 1 | 10 | 3 | 2 | 5 | 36 |

## TETANUS ANTITOXIN IN TREATMENT AND IMMUNIZATION.

While tetanus antitoxin has proved most efficacious in the prevention of tetanus, its employment as a curative agent has been much less successful. This failure to produce more uniformly good results is due chiefly to its too late administration. Insufficient dosage and the use of the subcutaneous method have also been important factors. While the subcutaneous use of antitoxin, which at first was the usual method employed, may prove of value in large doses given within the first few hours after the onset of symptoms, nevertheless it frequently fails because of slow absorption and the time required to reach the tissues of the central nervous system. In the light of our present knowledge, this method can be justified only by an inability of the physician, for one reason or another, to give an intravenous or an intraspinal injection. The latter method is being more and more recommended, and when employed before the disease has made too much headway has given much better results than the subcutaneous or even the intravenous.

From time to time since 1903 single cases or small series of cases have been reported in which the antitoxin has been given intraspinally. Thus Neugebauer[1] in 1905 reported 43 cases from Continental and American sources with a mortality of 22, or 51 per cent., and also 3 patients treated by him of whom 2 recovered. Many of these patients received also intravenous or subcutaneous injections or both. The results seemed to some observers to be better than when other methods were used without intraspinal dosage, but on the whole, this method of treatment has not made much headway. Experimental proof of the greater value of the intraspinal method has not until very recently been at all convincing. Permin, of Copenhagen, working on rabbits and dogs, showed that local tetanus could be prevented by the simultaneous injection of tetanus toxin intramuscularly and a dose of antitoxin given in the spine, whereas with the same dose of antitoxin given intravenously, local tetanus occurred. Four hours after the giving of the toxin alone neither method of preventing the occurrence of local tetanus was efficacious, and when nine hours were allowed to elapse between the giving of toxin and antitoxin, the animals could not be saved.

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[1] Wiener klin. Wchnschr., 1905, xviii, 450.

The following series of experiments were undertaken by Nicoll and us to determine to what extent tetanus antitoxin given in the spine has greater curative power, when the disease is actually established, than when given in the circulation. The comparative ineffectiveness of subcutaneous injections of tetanus antitoxin in developed cases is incidentally brought out. The use of guinea-pigs for this purpose has not been recorded so far as we know. They possess, however, a marked susceptibility to tetanus together, with comparative freedom from intercurrent diseases. The toxin in each case was inoculated intramuscularly into the thigh.

ADMINISTRATION AND RESULT OF TETANUS ANTITOXIN.

| No. | Weight. gms. | Condition of leg. | Method. | Amount. in units. | Result. |
|---|---|---|---|---|---|
| 116 | 290 | Fairly stiff | Control | ... | D 3 days. |
| 42 | 310 | Fairly stiff | Control | ... | D 3 days. |
| 296 | 250 | Slightly stiff | Heart | 100 | D 8 days. |
| 227 | 275 | Fairly stiff | Heart | 100 | D 4 days. |
| 399 | 300 | Fairly stiff | Heart | 100 | D 5 days. |
| 216 | 255 | Slightly stiff | Nerve | 200 | D 4 days. |
| 287 | 255 | Fairly stiff | Nerve | 200 | D 3 days. |
| 289 | 280 | Fairly stiff | Nerve | 200 | D 3 days. |
| 306 | 285 | Slightly stiff | Nerve | 200 | D 3 days. |
| 59 | 255 | Stiff | Spine | 10 | Discharged; normal. |
| 304 | 275 | Fairly stiff | Spine | 10 | Discharged; drags leg. |
| 321 | 320 | Fairly stiff | Spine | 10 | Discharged; drags leg. |

SIX GIVEN ANTITOXIN TWENTY-TWO AND A HALF TO TWENTY-THREE HOURS AFTER INOCULATION OF TOXIN.

| | | | | | |
|---|---|---|---|---|---|
| 102 | 300 | Stiff | Heart | 100 | D 5 days. |
| 10 | 325 | Stiff | Heart | 200 | D 4 days. |
| 272 | 350 | Stiff | Heart | 200 | D 4 days. |
| 263 | 285 | Stiff | Spine | 50 | Discharged; drags leg. |
| 123 | 325 | Stiff | Spine | 50 | D 5 days. |
| 294 | 350 | Stiff | Spine | 50 | Discharged; drags leg. |

This would seem absolutely conclusive of the superiority of the intraspinal method of giving antitoxin over the intravenous method. It will be noted that only those animals receiving the antitoxin in the spine were able to survive. This result is the more striking since the amount of antitoxin given them was only a fractional part of that given in the circulation. An attempt was made to give the antitoxin to four guinea-pigs intraneurally. Under an anesthetic the sciatic nerve of the affected limb was cut down upon and freed from the surrounding tissues, as much antitoxin as could be introduced into the nerve sheath injected, and the remainder intramuscularly directly along the course of the nerve. Owing to the small caliber of the nerve, most of the antitoxin passed to the deep intramuscular tissues. The animals lived no longer than those receiving subcutaneous injections.

In all of these experiments no guinea-pigs were discharged from observation until there was absolutely satisfactory evidence that the disease had long ceased to make any progress. While a number of the animals, perhaps most, on being discharged exhibited more or less stiffness in the inoculated leg lasting for weeks or even months, they were nevertheless in the best of health in other respects. This condition appears to be identical with that seen in human tetanus, in which the stiffness in different groups of muscles is often very prolonged even when the patients are perfectly convalescent.

Since beginning this work we have obtained records of twenty-four consecutive clinical cases of tetanus in which an intraspinal injection of antitoxin was given and eighteen patients recovered. In all of them in addition to the antitoxin used intraspinally, larger amounts were also given by other methods.

On experimental and clinical grounds the following recommendation for the treatment of tetanus with antitoxin would seem to be amply justified: In every case strongly suspected of being tetanus, from three to five thousand units of tetanus antitoxin should be given at the first possible moment intraspinally, slowly by gravity, and always, if possible, under an anesthetic. In order to insure its thorough dissemination throughout the spinal meninges the antitoxin should be diluted if necessary, to a volume of from 3 to 10 c.c. or more, according to the patient's age. When fluid is drawn off previously to the giving of the antitoxin, an amount of the latter somewhat less than that of the fluid withdrawn should be given. A number of cases of "dry tap" have been observed in the disease by those so expert in spinal puncture as to leave no room for doubt that the canal was properly entered. In such cases only a small amount of tetanus antitoxin should be injected (from 3 to 5 c.c.). In brief, tetanus antitoxins should be used in precisely the same way as antimeningitis serum.

**Results of the Use of Antitoxin for Immunization.**—The striking results which have been obtained, both in human and in veterinary practice, with the prophylactic injection of tetanus antitoxin, would seem to warrant the treating of patients with immunizing doses of serum—at least in neighborhoods where tetanus is not uncommon—when the lacerated and dirty condition of their wounds may indicate the possibility of a tetanus infection.

Splendid results have followed this practice in many places. It is the custom at many dispensaries in New York City and elsewhere to immunize all Fourth-of-July wounds by injecting 1000 units. None of these have ever developed tetanus. Even the few cases of human tetanus reported as occurring after single injections of antitoxin prove the value of immunizing injections, for the mortality was low. They teach, however, that where tetanus infection is suspected the antitoxic serum should be given a second and even a third time at intervals of seven days. In the European armies it was compulsory to give a serum injection to every wounded soldier at the first possible moment after the injury. A second injection was given ten days later. Since these regulations were adopted there have been almost no cases of tetanus.

## SERUM TREATMENT OF "GAS GANGRENE."

Both preventive and curative treatments of the wound condition known as "gas gangrene" (see p. 499) have been tried during the great war. Statistics in regard to their efficacy are still somewhat meagre but are on the whole favorable. In using serum as a curative agent the following four main factors should be considered:

1. The specificity of the serum against the particular infection. Thus the serums of Bull and Pritchett and Veillon produced only against the perfringens (B. Welchii) are not potent if the infection it caused by edemaciens or vibrion septique.
2. The time of application of serum. It should be injected as early as possible after the receipt of the wound. It is of no value when the infection has reached the stage of septicemia.
3. Thorough surgical procedure: all dead tissue should be removed and the wound thoroughly cleaned, so as to remove the favorable soil for the anaërobes and encourage phagocytosis by the healthy tissue.
4. Dosage and mode of injection. Enough serum should be given to neutralize the poison generated by the gas gangrene organisms. The injections are preferably intravenously, or may be given also intramuscularly around the wound, and repeated as often as necessary according to the condition of the patient and bacteriological findings.

Preventive serotherapy is by far the most satisfactory and is clearly outlined by some of the French surgeons (Delbet, Duval, Vaugher) in their recommendations to be made effective by the service of Santé. According to the latest reports the British and Americans adopted this procedure even before the French. These recommendations are as follows:

1. As gas gangrene develops in direct proportion with the delay of operation, exposure to wet and dirt on the battlefield and the kind of projectile, all those who by the nature of their wounds (muscular and vascular damage, pieces of clothing in the wound carried by the shrapnel, extensive fractures of the lower extremities) are most likely to develop gas gangrene should be injected with a preventive dose of the triple serum (edemaciens, perfringens, vibrion septique) at the first station from the battlefield together with antitetanus serum.

2. In no case should the operation be retarded on account of the injection. The serum treatment should be only an adjunct to surgical treatment.

3. As there is not enough serum to go around, all serums on the market should be tested for efficacy and accurately labelled as to against which organisms they are produced. In such a case many monovalent and bivalent sera could supplement each other. Unfortunately this systematic procedure has been adapted only shortly before the armistice was signed.

Favorable results of the preventive treatment have been reported by the French, English and American surgeons. Thus Duval and Vaugher give results on 381 cases which received preventive treatment. Only 4.7 per cent. developed gas gangrene as compared with from 15 per cent. to 18 per cent. of those (same category of cases) which were not subjected to preventive treatment.

Van Beuren[1] has just published a good summary of the subject.

## SERUM TREATMENT OF POLIOMYELITIS.

Only few investigators have claimed striking results for serum treatment in this disease. Two groups of cases have been treated. The first group were given either human convalescent serum or horse serum from horses inoculated with brain substance from infected monkeys and the results were not striking enough to warrant its extensive use in future epidemics.

The other group were those treated with horse serum obtained after the inoculation of the horses with streptococci obtained from cases of poliomyelitis by Rosenow[2] and Nuzum and Willy.[3] They both report markedly beneficial results. With our opinions of the etiology of the disease, it is difficult to believe that the serum was the cause of the improvement.

## ANTIPLAGUE SERUM.

Intravenous injections of 100 c.c. are given the sick. Persons exposed to infection should be given an immunizing dose of 20 c.c. This will give them a temporary immunity of about ten days. Those who are to come in later or prolonged contact with the disease should take Haffkine's vaccine.

## ANTIANTHRAX SERUM.

Actual cases receive intravenous injections of 50 to 100 c.c. of serum. These are to be repeated at end of twelve hours. The serum from horses which have been hyperimmunized confers immunity upon animals injected with it. Sclavo found that he could save animals injected twelve hours after being infected. Serum treatment has been used frequently in Europe. The dosage is 50 to 100 c.c. It is given intravenously in bad cases and either intravenously or intramuscularly in slight cases. The serum is repeated in twelve hours and then daily.

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[1] Jour. Am. Med. Assn., 1919, lxxiii, 239.

[2] Rosenow: Jour. Am. Med. Assn., 1918, lxxi, 433.

[3] Jour. Am. Med. Assn., 1917, lxix, 1247.

## CHAPTER XLVIII.

# THE BACTERIOLOGICAL EXAMINATION OF WATER, AIR, AND SOIL. THE CONTAMINATION AND PURIFICATION OF WATER. THE DISPOSAL OF SEWAGE.

### EXAMINATION OF WATER.

THE bacteriological examination of water is undertaken for the purpose of discovering whether any pathogenic bacteria are liable to be present. The determination of the number of bacteria in water was for a time considered of great importance, then it fell into disrepute, and the attempt was made to isolate the specific germs of diseases which were thought to be water-borne. At first these attempts seemed very successful in that supposed typhoid bacilli and cholera spirilla were found. Further study revealed the fact that there were common water and intestinal bacteria which were so closely allied to the above forms that the tests applied did not separate them. When proper identification was carried out the very great majority of the suspected organisms were found to be non-pathogenic. The improbability of getting typhoid bacilli from suspected water except under unusually favorable conditions caused a return to the estimation of the number of bacteria in water and above all to the estimation of the number of intestinal bacteria. It is known that the group of colon bacilli have a somewhat longer existence than the typhoid bacilli, and as the colon bacilli come chiefly or wholly from the intestinal passages of men and animals, it was fair to assume that typhoid bacilli, dysentery or other pathogenic bacteria could not occur from fecal pollution without the presence of the colon bacillus except in rare cases.

During the past few years the attention of sanitarians has been seriously devoted to the interpretation of the presence of smaller or larger numbers of colon bacilli in water, until at present, upon the quantitative analysis (measuring, within certain limits, decomposing organic matter) and the colon test (indicating more specifically pollution derived from intestinal discharges of man or animals) the bacteriological analysis of water is based. The determination of the number of bacteria is also of value.

**Technic for Quantitative Analysis.**—The utmost care is necessary to get reliable results. A speck of dust, a contaminated dish, a delay of a few hours, an improperly sterilized agar or gelatin, a too high or too low temperature, may introduce an error or variation in results which would made a reliable test impossible.

**Collection of Samples.**—The small sample taken must represent the whole from which it was drawn. If a brook water, it must be taken some distance from the bank; if from a tap, the water in the pipes must first be run off, for otherwise the effect of metallic substances will invalidate the results; if from lake or pond, the surface scum or bottom mud must be avoided, but may be examined separately. The utensils by which the water is taken should be of a good quality of glass, clean and sterile. From a brook the water can be taken directly into the bottle, the stopper being removed while it fills, avoiding the surface film and its attending excessive numbers of bacteria; from a river or pond it can be taken from the bow of a small boat, or from a bottle properly fastened on the end of a pole so as to avoid contamination; from a well a special apparatus has been devised by Abbott, where a bottle with a leaded bottom is so held that when lowered to the proper depth a jerk will remove the cork and allow the bottle to fill. The same device or another accomplishing the same purpose can be rigged up readily by anyone. The sample of water should be tested as soon as possible, for the bacteria immediately begin to increase or decrease. In small bottles removed from the light, predatory microörganisms and many bacteria begin to increase, and among these are the members of the colon group. Thus, the Franklands record a case in which in a sample of well water kept during three days at a moderate temperature the bacteria increased from 7 to 495,000; while Jordan found that in a sample the bacteria in forty-eight hours fell from 535,000 to 54,500. In a sample we kept at room temperature the colon bacilli during twenty-four hours increased from 10 to 100 per c.c. The only safe way to prevent this increase is to plate and plant the water in fermentation tubes within a space of one or two hours or to keep it at a temperature under 5° C. (41° F.). If it cannot be kept cold, it is far better to make the cultures in the open field or in a house rather than to wait six to twelve hours for the conveniences and advantages of the laboratory.

The third matter of great importance is the adding of proper amounts of water to the broth in the fermentation tubes and the media for planting. Usually 1 c.c., 0.1 c.c., and 0.01 c.c. are added to the fermentation tubes and to 10 c.c. of the melted standard nutrient agar or gelatin. If possible, duplicate tests should always be made. When it is desired to know whether colon bacilli are present in larger amounts than 1 c.c., quantities as great as 10 to 100 c.c. can be added to bouillon, and then after a few hours 1 c.c. added to fermentation tubes. Less than twenty colonies and more than two hundred on a plate give inaccurate counts, the smaller number being too few to judge an average and the larger number interfering with each other. When as many as 10,000 colonies develop in the agar contained in one plate, it will be found that there will develop in a second plate containing but one-tenth the amount of water from 20 to 50 per cent. as many colonies. This shows that the crowding of the colonies had prevented the growth or caused a fusion of all but one-fifth to one-half of them.

The chemical composition of the medium on which the bacteria are grown affects the result of the analysis. Nutrient 1.5 per cent. agar gives slightly lower counts than gelatin, but on account of its convenience in summer and its greater uniformity it is being more and more generally used for routine quantitative work. A uniform standard is a necessity to secure comparability of results. (See Media for Water.) At best only a certain proportion of bacteria develop, and it is only important that our counts represent a section through the true bacterial flora which fairly represents the quick-growing sewage forms. Comparability is the vitally essential factor.

The temperature at which the bacteria develop is of great importance, and they should be protected from light. The access of oxygen which prevents the growths of anaërobes must also not be forgotten. As a rule, the plate cultures are developed at two temperatures, for four days at 20° to 21° C., and for twenty-four to forty-eight hours at incubator temperature. Some bacteria do not develop colonies in four days, but these are neglected. The number of bacteria growing at room temperature is usually much greater than those growing at 37° C. As all the intestinal groups of bacteria grow at body temperature, while many of the water types do not, some investigators believe it important to develop the bacteria at both temperatures so as to compare the results. We have not found this to be of any advantage when tests are also made for the colon group of bacilli.

The lactose broth, with indicator, is placed at 37° C. for growing the colon bacilli. The fermentation tubes not showing gas are recorded as negative and usually discarded. Those showing gas are suspected to contain colon bacilli. To a number of tubes containing melted litmus-lactose agar at about 44° C., are added 1, 0.1, and 0.01 loop of the culture fluid. Plates are poured and the whole placed in the incubator. The *Bacillus coli* ferments lactose and thus produces acid, so that if colon bacilli are present we have a number of red colonies on a blue field. Later, if many colon bacilli were present, the whole medium becomes acid. At forty-eight hours, on account of alkali being produced by the formation of $NH_3$, the blue may return. If after inspection red colonies are seen, four or five are picked and planted into lactose bouillon and other media. Litmus lactose agar is frequently used for the original plating of water samples, the absence or presence of acid-producing colonies being thus immediately noted. The results of these tests are all more or less presumptive evidence of the bacilli belonging or not belonging to the intestinal colon bacillus group. Unfortunately there are some types of bacilli growing in the soil which resemble them. If it is necessary to be more accurate, the colon-like cultures should be subjected to the Vosges reaction (page 146), and should be kept for one month at 20° C. in gelatin before a decision is made. Colon bacilli do not liquefy gelatin nor give the Vosges reaction (page 146). There are a few colon-like bacilli in the intestinal tract that give the Vosges reaction. For a more complete understanding of the technic and the interpretation of results of the bacteriological examination of water see *Elements of Water Bacteriology*, by Prescott and Winslow.

For the characteristics of the colon bacilli the Massachusetts State

Board of Health uses six media—gelatin, lactose agar, dextrose broth, milk, nitrate solution, and peptone solution, determining, respectively, absence of liquefaction, production of gas turbidity, coagulation without liquefaction of the coagulum, production of nitrite, and of indol.

Lactose-bile-peptone solution has been much used. In badly contaminated waters this has a distinct advantage in that the bile inhibits many varieties of bacteria more than those of the colon-typhoid group. In good waters the results are very similar from the lactose-peptone and lactose-blue-peptone solutions.

**Significance of the Colon Bacillus.**—The colon test has been received by the majority of engineers and practical sanitarians with great satisfaction, and has been applied with confidence to the examination not only of water, but of shell-fish and other articles of food as well. On the other hand, some have denied its value. Bacteriologists have found bacilli like certain members of the colon group in apparently unpolluted well water. The discovery that animals have colon bacilli identical in the usual characteristics studied with those of man has complicated matters. Thus a fresh hillside stream may be loaded with colon bacilli from the washings of horse or cow manure put on the fields through which it runs or polluted by a stray cow or horse. Swine, hens, birds, etc., may contaminate in unsuspected ways. The number of colon bacilli rather than their presence in any body of surface water is therefore of importance. In well and spring water the presence of colon bacilli indicates contamination. The absence of colon bacilli in water proves its harmlessness so far as bacteriology can prove it. When the colon bacillus is present so as to be isolated from 1 c.c. of water in a series of tests, it is reasonable proof of animal or human pollution and the conditions should be investigated. Ten colon bacilli in 1 c.c. indicates serious pollution. Surface waters from inhabited regions will always contain numerous colon bacilli after a heavy rainstorm or shower. The washings from roads and cultivated fields contain necessarily large numbers. Winslow reports that in only two out of fifty-eight samples of presumably non-polluted well water did he get colon bacilli in the 1 c.c. samples. Even in twenty-one stagnant pools he only found colon bacilli in five of the 1 c.c. samples.

The experience of all who have studied the subject practically is that in delicacy the colon test surpasses chemical analysis; in constancy and definiteness it also excels the quantitative bacterial count. All these tests must, however, be supplemented by inspection.

**Interpretation of the Quantitative Analysis.**—The older experimenters attempted to establish arbitrary standards by which the sanitary quality of water could be fixed automatically by the number of germs alone. This has been largely given up. Dr. Sternberg considers that a water containing less than 100 bacteria is presumably from a deep source and uncontaminated by surface drainage; that one with 500 bacteria is open to suspicion; and that one with over 1000 bacteria is presumably contaminated by sewage or surface drainage. Even this conservative opinion must be applied with caution.

The source of the sample is of vital importance in the interpretation; thus, a bacterial count which would condemn a spring or well might be normal for a river. In woodland springs and lakes several hundred bacteria per cubic centimeter are frequently found. In lakes the point at which the sample is taken it of great importance, as the bacterial count varies with the distance from the shore and with the depth. The weather also is an influence, since the wind causes currents and waves which stir up the bottom mud, bringing up organisms which have been sedimented. Rains greatly influence streams by flooding them with surface water, bringing a huge number of bacteria at times. The season of the year is an important factor. The counts are highest in the winter and spring months, and lower from April to September.

The following figures illustrate this point:

| Water. | Observer. | Year. | Jan. | Feb. | Mar. | April. | May. | June. |
|---|---|---|---|---|---|---|---|---|
| New York City tap water | Houghton | 1904 | 890 | 1100 | 650 | 240 | 350 | 370 |
| New York City | Noble | 1916 | 13 | 17 | 50 | 33 | 6 | 13 |
| Boston tap water | Whipple | 1892 | 135 | 211 | 102 | 52 | 53 | 86 |
| Merrimac River tap water | Clark | 1899 | 4900 | 5900 | 6300 | 2900 | 1900 | 3500 |

The winter and spring increases are not exceptions to the rule that high numbers indicate danger, but an indication of its truth; for it means a melting of the snow and a flow of surface water into the streams without the usual filtering soil percolation. A number of severe epidemics of typhoid fever have been produced in this way. It is only the fact that typhoid fever is at a minimum in winter that prevents more frequent pollution. Although, as a rule, a series of tests are necessary to pass judgment on a water, a single test may be very important. A large increase in the number in tap water a day after a storm points unerringly to surface pollution, and if towns exist in the watershed, to street and sewer pollution. The Croton water frequently jumps from hundreds to thousands after such a storm. The low bacterial content in 1916 is due to the addition of chlorine to the water after its leaving the reservoir.

In a typhoid epidemic at Newport, Winslow reports that a test of the water supply showed but 334 bacteria per cubic centimeter, but one from a well showed 6100. The suspicion aroused was justified by finding all the typhoid cases had gotten water from this well.

The study of the bacterial effluent from municipal water filters is the only way in which the efficiency of the filter and the accidents which occur can be determined. In Germany these regular tests are obligatory. The filter should remove about 99 per cent. of the bacteria. Elaborate studies have recently been made of the exact distribution of streams of sewage in bodies of water into which they flow, their disappearance by dilution and sedimentation, and their removal by death. Under peculiar conditions bacteria in water may increase for a time, but here the prevailing bacteria belong almost exclusively to one type.

**Streptococci in Sewage.**—The varieties of streptococci found most often in polluted water correspond to the streptococci described by Houston. In some water in which these are found no *B. coli* have been found and there is considerable doubt in such cases as to whether the streptococci imply serious pollution. The streptococci remain alive longer than the colon bacilli. In England the examination for streptococci in water is being less regularly done than formerly.

**Other Bacteria.**—Most of the bacteria which develop in the intestines of man and animals necessarily occur in polluted water, and an examination for some of these has been advocated by many, such as the *B. (enteritidis) sporogenes*, other anaërobic spore formers, the various members of the typhoid-colon group, and the proteus group.

**Isolation of the Typhoid Bacillus from Water.**—If it were possible to readily obtain the typhoid bacilli from water, when they were present in small numbers, its examination for that purpose would be of much greater value than it is now; but we have to remember that we can only examine at one time a few cubic centimeters of water by bacteriological methods, and that although the typhoid bacilli may be sufficiently abundant in the water to give, in the quantity that we ordinarily drink, a few bacilli, yet it must be a very lucky chance if they happen to be in the small amount which we examine. Still further, although it is very easy to isolate typhoid bacilli from water when they are in considerable numbers, yet when they are a very minute proportion of all the bacteria present it is almost impossible not to overlook them. Many attempts have been made to devise some method by which the relative number of the typhoid and other parasitic bacteria present in water could be increased at the expense of the saprophytic bacteria. Thus, to 100 c.c. of water 25 c.c. of a 4 per cent. peptone nutrient bouillon is added, and the whole put in the incubator at 38° C. for twenty-four hours. From this, plate cultures are made. As a matter of fact, the typhoid bacillus is rarely found, even in specimens of water where we actually know that it is or has been present because of cases of typhoid fever which have developed from drinking the water. From these facts we must consider our lack of finding the bacillus in any given cases as absolutely no reason for considering the water to be free from danger. Another serious drawback to the value of examinations for the typhoid bacillus is that they are frequently made at a time when the water is really free from contamination, though both earlier and later the bacillus was present. It is hardly worth while, therefore, except in careful experimental researches, to examine the water for the typhoid bacillus, but rather study the location of the surrounding privies and sources of contamination. A number of observers, resting on the agglutination test, have thought they have isolated typhoid bacilli from the soil and water, but these investigators had not considered sufficiently the matter of group agglutinins, and their results are not trustworthy.

## CONTAMINATION AND PURIFICATION OF DRINKING WATERS.

Brook water and river water are contaminated in two ways: through chemicals, the waste products of manufacturing establishments, and through harmful bacteria by the contents of drains, sewers, etc., the latter method being far the more dangerous.

When water, which has been soiled by waste products of manufactories only, becomes so diluted or purified that the contamination is not noticeable to the senses and shows no dangerous products on chemical analysis, it is probably safe to drink. When sewage is the contamination, this rule no longer holds, and there may be no chemical impurities and no pathogenic bacteria found, and yet disease be produced. That river water which has been fouled by sewage will by oxidation, dilution, sedimentation, action of sunlight, and predatory microörganisms, become greatly purified is an indisputable fact. The increase in bacteria which occurs from contamination is also largely or entirely lost after ten to twenty miles of river flow. Nevertheless, the history of many epidemics seems to show that a badly contaminated river is never an absolutely safe water to drink, although with the lapse of each day it becomes less and less dangerous, nor will sand filter-beds absolutely remove all danger. These statements are founded upon the results of numerous investigations; thus the marked disappearance of bacteria is illustrated by the following: Kummel found below the town of Rosbock 48,000 bacteria to the cubic centimeter; twenty-five kilometers farther down the stream only 200 were present—about the same number as before the sewage of Rosbock entered. On the other hand, the doubtful security of depending on a river purification is proved by such experiences as the following: In the city of Lowell, Massachusetts, an alarming epidemic followed the pollution of the Merrimac River three miles above by typhoid feces, and six weeks later an alarming epidemic attacked Lawrence, nine miles below Lowell. It is estimated that the water took ten days to pass from Lowell to Lawrence and through the reservoirs. Typhoid bacilli usually die in river water in from three to ten days, but they may live for twenty-five days in other water; the Lawrence epidemic is easily explained. Newark-on-Trent, England, averaged 75 cases a year from moderately well-filtered water and only 10 when it was changed to deep-well supply.

**Purification of Water on a Large Scale.**—For detailed information on this subject the reader is referred to works on hygiene. Surface waters, if collected and held in sufficiently large lakes or reservoirs, usually become so clarified by sedimentation, except shortly after heavy rains, as to require no further treatment so far as its appearance goes. The collection of water in large reservoirs allows not only the living and dead matter to subside, but allows time also for the pathogenic germs to perish through the influence of light as well as of antagonistic bacteria and other deleterious influences, such as sand, or mechanical coagulant. Filtration of water exerts a very marked purification, taking out 99.8 per cent. of the organisms in those best constructed and at least 95 per cent. in those commonly used in cities. The construction of filters is too large a subject to enter on minutely here; sand filters consist, as a rule, of several layers, beginning with fine sand, and then smaller and larger gravel, and finally rough stones.

A certain time elapses before the best results are obtained; this seems to wait for the formation of a film of organic material on the sand, which is full of nitrifying bacteria. Even the best filters only greatly diminish the dangers of polluted water. Spring water and well water are, in fact, filtered waters.

Water which is subject to serious pollution must be submitted to a preliminary purification before it can be considered a suitable source for a drinking-water supply. The means employed for its purification depend to a large extent upon the character of the water and the nature of the pollution. Filtration through slow sand filters, three to five feet in depth, removes 98 to 99.5 per cent. of the bacteria and organic matter; so that effluents from the best constructed sand filtration beds constitute safe and reliable drinking waters. Five hundred thousand to one or two million gallons, depending somewhat upon the extent of pollution and the fineness of the sand, can be filtered daily per acre. Only the surface of the sand filter becomes in any way clogged and as thin a layer as can be scraped off is removed one or more times a month. This surface sand is washed with clean water and several scrapings replaced at one time. Sand filtration beds are very widely used abroad and are coming into extensive use in this country. The filter-beds at Lawrence, Mass., have been used over ten years with marked success; when properly managed, they render the highly polluted Merrimac River a fairly safe drinking water; the filter-beds there are scraped about thirteen times a year.

Mechanical filtration plants find considerable favor where clarification as well as bacterial purification is desired. A coagulant such as sulphate of aluminum is employed and forms in the water a flocculent precipitate which carries down with it all suspended matter; 125,000,000 or more gallons of water can be filtered on an acre daily, but the filters must be washed daily by reversing the flow and cleansing the clogged filter with a stream of the purified water. Chlorinated lime when added to drinking water to the extent of one-eighth to one-twelfth of a grain per gallon will destroy all intestinal bacteria of the typhoid-colon group within a few hours. This is a very useful means of purification. It does not injure the water and is being used very extensively.

Under special conditions other methods, such as the passage of ozone, have proved successful.

**Domestic Purification.**—Water which requires private filtering should not be supplied for drinking purposes. Unhappily, however, it often is. Domestic filters may be divided, roughly, into those for high and low pressure. The former are directly connected with the water main, while the others simply have the slight pressure of the column of water standing in the filter. Many household filters contain animal charcoal, silicated carbon, etc., either in a pressed condition or in one porous mass. These filters remove much of the deleterious matter from the suspected waters, but the majority cannot be depended upon to remove all bacteria. Even those which are equipped for self-cleansing become foul in a little while, and, if not cleaned, unfit for use. The best of the filters are of porous stone, such as the Poulton-Berkefeld and Pasteur filters. These yield a water, if too great a pressure is not used, almost absolutely free from bacteria, and if they are frequently cleansed they are reliable. A large Berkefeld filter will allow sixty gallons of water to pass per hour. The Pasteur filter is more compact and slower. From the best Pasteur filters sterile water may be passed for two or three weeks; from the Berkefeld usually only a few days. A single typical low-pressure filter is that of Bailey Denton. The upper compartment contains the filtering material, which may be sand or charcoal, and is fed from a cistern or hydrant. After a certain quantity of water has passed in, the supply is automatically cut off until the whole amount is filtered. A fairly efficient filter is the following: Take a large-sized earthenware pot and plug the hole in the bottom with a cork, through which pass a short glass tube. Upon the bottom place an inch of small pieces of broken flower-pot; upon this a couple of inches of well-washed small gravel, and upon this six to twelve inches of well-washed fine, sharp sand. Cover the same with a piece of filter-paper and hold this down with a few small stones. Mount the pot on a tripod, and it is ready for use. The paper prevents the sand being disturbed when water is added, and as it also holds most of the sediment, this can be readily removed. Every few months the sand can be washed and replaced. Animal charcoal is not a good substance for permanent filters, as bacteria grow well in it. Whenever water is suspected, and there is any doubt as to the filters, it should be boiled for ten minutes; this will destroy all bacteria. This precaution should always be taken in the presence of typhoid fever and cholera epidemics.

## THE DISPOSAL OF SEWAGE.

The disposal of sewage is becoming a vital question with all towns and cities which are not situated near salt-water outlets, since the present tendency in legislation is to compel such towns to dispose of their waste so that it shall not be a menace to drinking-water streams, destructive to fisheries, or a nuisance to harbors.

Methods of sewage purification depend upon the character of the sewage and the kind of effluent desired.

Two hundred thousand gallons of crude sewage may be filtered upon an acre of land daily and an effluent obtained which will compare favorably in every way known to the chemist and bacteriologist with the best mountain springs. This is, however, a slow process, and it is rare that such a pure effluent is required. Similar results may be obtained by utilizing the septic-tank method, running the sewage from the septic tank to contact beds and thence to sand filter-beds; where, because of the partial "self-purification of the sewage" in the septic tank and contact beds, 2,500,000 gallons of sewage can be filtered daily on an acre of surface. In this process less land is required and both these effluents can be safely turned into drinking-water streams.

If, however, a merely non-putrescible effluent is required, one which, though high bacterially, will not be offensive in any way, or subject to further decomposition, it may be obtained by passing crude sewage to septic tanks, thence to double contact beds, the resulting effluent having merely an earthy, humus-like odor and being non-putrescible.

Where acid wastes, tannery wastes, dyestuffs, etc., from various factories enter into sewage, its disposal becomes a more complicated problem and chemical precipitation by the use of lime or other chemicals is generally employed for such sewage purification, which at best is only partial and is sometimes supplemented by sand filtration.

**Sea Water.**—This is only feebly bactericidal. The salty tidal waters of rivers allow typhoid bacilli and other members of the typhoid colon group to live for a number of days.

## BACTERIA IN AIR—EXAMINATION OF AIR.

Saprophytic bacteria are always present in considerable numbers in the air except far out at sea or on high mountains. They reach the air from the earth's surface and are most plentiful nearest to it. They are more abundant where organic matter abounds and in dry and windy weather.

The air is kept constantly in motion by winds so that fine particles are constantly being carried into it from the ground, especially in an inhabited area with its dusty streets. The rays of sunlight visibly reveal these particles to us. The bacteria in the dust of the fields and streets are carried along with these dust particles. They are usually the harmless soil bacteria or the almost equally harmless intestinal bacteria of animals. After a storm few bacteria are in the air, while on a dry windy day many thousands exist in a cubic meter. In warm weather rain carries down the bacteria of the air. The number of bacteria in the air of the country are much less than in the city air. Forests decrease the number of bacteria.

On high mountains and on the sea far from land, bacteria are very scarce. The bacteria that multiply in the soil of street and country are almost entirely saprophytic types. Sunlight and drying rapidly destroy bacteria. In dwellings the bacterial content depends on many factors, of which the chief are the opening of windows to the outside dust-laden air, the cleanliness of the dwelling, and the amount of stirring up of the dust by sweeping. It is almost impossible to separate the effect of the bacteria that we inhale from that of the dust particles which they accompany. Both probably act as slight irritants and so predispose to definite infections.

Except in the air in direct contact with men and animals pathogenic bacteria are present in the air only under exceptional circumstances and usually as spores, such as those of anthrax bacilli from the dust from the wool and hides of infected animals or of tetanus bacilli from the infected manure. The practical results obtained from the examination of air for pathogenic bacteria have been slight. We know that at times they must be in the air, but unless we purposely increase their numbers they are so few in the comparatively small amount of air which it is practicable to examine that we rarely find them. It is established that in loud speaking, in coughing and sneezing many bacteria from the larynx, fauces and lips are expelled. Examination of dust in hospital wards and sick rooms, in places where only air infection was possible, has occasionally revealed tubercle bacilli and other pathogenic bacteria. Although very light they generally settle to the ground. It is now thought that the factor of air infection in the spread of infectious diseases is of very little importance, except when recent droplet infection has occurred. With the tiny droplets of mucus most of these bacteria die quickly and do not disseminate, while alive, far from the place of their origin.

The simplest method of searching for the varieties of bacteria in the air and their number in any place is to expose to the air for longer or shorter periods nutrient agar spread upon the surface of the Petri dish. After exposure the plates are either put in the incubator at 37° C., or kept at room temperature. When it is desired to obtain the pathogenic bacteria a little rabbit's blood is smeared over the agar. The more careful quantitative examination is made by drawing a given quantity of air through tubes containing sterile sand, which is kept in place by pieces of metal gauze. When the operation is completed the sand is poured into a tube containing melted nutrient gelatin or nutrient agar, and after thorough shaking, the mixture is poured into a Petri dish and the bacteria allowed to develop, either at 37° or 23° C., according as the growth of the parasitic or saprophytic varieties is desired. Instead of nutrient agar or gelatin, blood agar, ascitic broth or animals may be inoculated. Such examinations are occasionally made of the air of theatres, crowded streets in cities, etc. They give usually the number of non-pathogenic bacteria only.

## BACTERIOLOGICAL EXAMINATION OF THE SOIL.

The subject from its agricultural side is considered in Chapter LI. Specimens of deep soil can be gathered in sterile, sharp-pointed, sheet-iron tubes. Through the examination, we wish to learn either the number of bacteria or the important varieties of bacteria present. To estimate the number, small fractions of a gram are taken and planted in nutrient agar or in special media contained in Petri dishes. Anaërobic as well as aërobic cultures should be made.

According to Houston, uncultivated sand soil averages 100,000 bacteria per gram, garden soil 1,500,000, and sewage-polluted 115,000,000. The most important bacteria to be sought for are bacilli of the colon group and streptococci. Both of these suggest fairly recent excremental pollution. Tetanus spores may also be found.

The period during which typhoid bacilli remain alive in soil is variable, since it depends on so many unknown factors and differs so in different places. The typhoid bacilli probably rarely increase in the soil and probably rarely survive a month in it. The main danger of soil bacteria is their being washed into water supplied by rains or carried to them by the wind.

# CHAPTER XLIX.

# THE BACTERIOLOGY OF MILK IN ITS RELATION TO DISEASE.

From the standpoint of the dairy many of the different varieties of bacteria found in milk are of importance which have little or no medical interest. We have space here only to consider the bacteriology of milk so far as it is related to health and disease. The fermented milks were considered in Chapter XXI. The saprophytic bacteria taken collectively have importance because one can determine from their number something as to the care taken in handling the milk and also because, when numerous, they produce chemical changes in the milk which are harmful for infants. The bacilli of the colon group are of little more importance than any of the saprophytic types, as in the case of milk they simply indicate pollution from the cow which is no more or less harmless than other forms.

**Numerical Estimation of Bacteria.**—The number of bacteria in a cubic centimeter of milk is usually estimated from the colonies developing in standard nutrient agar plate cultures during a period of forty-eight hours when kept at a temperature of 37° C. This allows in market milk in which bacteria have developed at low temperature only a certain proportion of the varieties of bacteria to develop colonies. Sometimes fully twice as many colonies develop at 20° to 27° C. during three to five days as at 37° C. for two days. The advantages of the shorter time and of uniformity have led to the adoption of the technic given in Part I. Any culture method necessarily underestimates their number, as many of the bacteria remain after vigorous shaking in pairs or small groups. In order to overcome this and also to note the morphological types, the direct microscopic examination of smears of the sediment has been urged. A great practical objection to this is that, if a heated milk is examined, the dead as well as the living bacteria are counted, for no satisfactory method has yet been found by means of stains to differentiate between them. It is true that many varieties of bacteria stain less intensely after heating but others do not. This method has, however, great advantages at the creamery or farm in that one can immediately tell whether a sample has few or many bacteria and also note the presence of streptococci and leukocytes. The microscopic count gives from two to ten times as many bacteria as the routine culture method when the individual bacteria are counted. When clumps of bacteria are given the same value as single bacteria the microscopic counts agree closely with the colony counts. The important thing in the estimation of the number of bacteria in milk is to use standard methods so that uniform results will be obtained by all careful technicians.

**Smear Method for Direct Examination of Milk.**—The Prescott-Breed[1] method is the most accurate. That of Slack is also used. (See below.)

**Advantages and Disadvantages of the Direct or Microscopic Method of Milk Analysis.—Advantages.**—1. The counting of each organism seen, while difficult and time-consuming, makes it possible to ascertain approximately the actual number of bacteria in milk. The counting of the clumps as one organism approximates the colony count, but loses the value of an exact estimation.

2. It eliminates the greater portion of glassware required at present in the plate method, the preparation of agar, etc., which lessens the initial cost.

These advantages, however, accrue mainly to the dealers employing a bacteriologist to make the milk analyses at the source.

**Disadvantages.**—The disadvantages of the direct method are: 1. The very small quantity of milk used for counting leads to inaccuracy.

2. That as a resultant of the foregoing facts large factors have to be used in estimating the bacterial content of milk, with the consequent introduction of large factors of error.

3. In estimating the bacterial content at all accurately of a very good milk, large numbers of fields have to be examined with a consequent expenditure of time, which increases the cost.

4. The individuality of the counter enters more largely into this method than in the plate method, and a better trained bacteriologist is necessary.

| Samples graded on colony count, bacterial content per c.c. | No. of samples. | Average plate count. | Average microscopic count of individual organisms. | Ratio. |
|---|---|---|---|---|
| Less than 50,000 | 594 | 14,491 | 92,034 | 1:6.29 |
| 50,000 to 300,000 | 133 | 103,075 | 708,681 | 1:6.87 |
| 300,000 to 1,500,000 | 42 | 648,452 | 5,254,018 | 1:8.1 |
| 1,500,000 to 5,000,000 | 13 | 2,538,461 | 21,541,534 | 1:8.48 |
| 5,000,000 or over | 8 | 6,575,000 | 27,519,531 | 1:4.18 |
| Total | 790 | 171,181 | 1,100,997 | 1:6.43 |

Prescott and Breed explain their smear method briefly as follows: The sample of milk to be examined is shaken thoroughly and 0.01 c.c. is withdrawn by means of a specially constructed capillary pipette. The milk so obtained is spread evenly over an area of one square centimeter on an ordinary glass slide. These areas may be easily determined by placing the glass slide over paper or glass on which areas of this size have been accurately ruled out. More satisfactory results can be obtained by using circular instead of square areas. Duplicate smears should be made on the same slide. The milk is then dried with gentle heat, the fat dissolved out with xylol or other fat solvent, the smear again dried, immersed in alcohol for a few minutes to fix the film, again dried and stained with methylene blue or other stain. Alkaline stains or others which attack the casein and loosen the smear must be avoided. The glassware used must be cleansed but need not be sterilized, as the bacteria have no chance to increase in number.

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[1] Centralbl. f. Bakteriol., Parasiten u. Infectionskrankheiten, 1911, Abt. II, Nos. 16, 18, xxx, 337.

The counting of the bacteria is done by a microscope and an oil-immersion lens. If the diameter of the field is so adjusted, by means of the draw tube, that it equals 0.16 mm., then each field of the microscope covers approximately one-five-thousandth (0.0002) of a square centimeter. On this basis each bacterium seen in a field taken at random represents 500,000 per c.c. if they are evenly distributed. However, it is impossible to distribute them evenly so that at least 110 fields of the microscope should be counted when there are many bacteria, 50 fields when the number is moderate and 100 fields if accurate results are required. The total number of bacteria seen in 10 fields multiplied by 50,000 or the total number seen in 100 fields multiplied by 5000 gives approximately the total number of bacteria per cubic centimeter. The counts vary considerably when individual bacteria are counted, even when made by the same examiner, because of one or more large clumps being in one series of fields and not in another. Even when clumps are counted as one the distribution varies in the different fields.

COMPARISON BETWEEN MICROSCOPIC COUNTS BY DIFFERENT EXAMINERS.

| Examiner. | | |
|---|---|---|
| A | Count of individual bacteria | 164,000 |
| | Count of clumps | 65,000 |
| B | Count of individual bacteria | 29,000 |
| | Count of clumps | 8,000 |
| C | Count of individual bacteria | 133,000 |
| | Count of clumps | 38,000 |

**Varieties.**—Bacteria in milk can be divided into two great groups—those which get into the milk after it leaves the udder and those which come from the cow. The first group comprises bacteria from dust, hands, milking pails, strainers, etc.

The extraneous bacteria are of importance because they indicate the conditions under which the milk was produced and cared for and because they produce changes in the chemical composition of the milk when they have developed in great numbers. The number of bacteria in any sample of milk depends on three factors: the number deposited in the milk from the cow's udder, from the air, and utensils; the time during which they have developed, and the temperature at which the milk has stood. The last is perhaps the most important factor. The attempt was made during a period of one year to connect illness in infants and children with special varieties of saprophytic bacteria in milk, but with negative results.

From the milks altogether 239 varieties of bacteria were isolated and studied. These 239 varieties, having some cultural or other differences, were divided into 31 classes, each class containing from 1 to 39 more or less related organisms.

As to the sources of bacteria found in milk, we made sufficient experiments to satisfy us that they came chiefly from outside the udder and milk ducts.

Bacteria were isolated from various materials which under certain conditions might be sources of contamination for the milk, and the cultures compared with those taken from milk. Thus there were obtained from 20 specimens of hay and grass, 31 varieties of bacteria; from 15 specimens of feces, manure, and intestinal contents, 28 varieties; from 10 specimens of feed, 17 varieties. Of these 76 varieties there were 42 which resembled closely those from milk—viz., 11 from grass or hay; 26 from manure; 5 from feed.

During the investigation a number of the varieties isolated from milk were shown to be identical with types commonly found in water.

From the few facts quoted above and from many other observations made during the course of the work it would seem that the term "milk bacteria" assumes a condition which does not exist in fact. The expression would seem to indicate that a few varieties, especially those derived in some way from the cow, are commonly found in milk, which forms having entered the milk while still in the udder or after its withdrawal, are so well fitted to develop in milk that they outgrow all other varieties.

As a matter of fact it was found that milk taken from a number of cows, in which almost no outside contamination had occurred, and plated immediately, contained, as a rule, very few bacteria, and these were streptococci, staphylococci, and other varieties of bacteria not often found in milk sold in New York City; the temperature at which milk is kept being less suitable for them than for the bacteria which fall into the milk from dust, manure, etc. A number of specimens of fairly fresh market milk averaging 200,000 bacteria per cubic centimeter were examined immediately, and again after twelve to twenty-four hours. In almost every test the three or four predominant varieties of the fresher milk remained as the predominant varieties after the period mentioned.

The above experiments seem to show that organisms which have gained a good percentage in the ordinary commercial milk at time of sale will be likely to hold the same relative place for as long a period as milk is usually kept. After the bacteria pass the ten or twenty million counts a change occurs, since the increasing acidity inhibits the growth of some forms before it does that of others. Thus some varieties of the lactic acid bacteria can increase until the acidity is twice as great as that which inhibits the growth of many bacteria. Before milk reaches the curdling point, the bacteria may have reached over a billion to each cubic centimeter. For the most part specimens of milk from different localities showed a difference in the character of the bacteria present, in the same way that the bacteria from hay, feed, etc., varied. Even the intestinal contents of cows, the bacteriology of which might be expected to show common characteristics, contained, besides the predominating colon types, other organisms, which differed widely in different species and in different localities. Cleanliness in handling the milk and the temperature at which it had been kept were also found to have a marked influence on the predominant varieties of bacteria present.

**Pathogenic Properties of the Bacteria Isolated.**—Intraperitoneal injection of 2 c.c. of broth or milk cultures of about 40 per cent. of the varieties tested caused death. Cultures of most of the remainder produced no apparent deleterious effects even when injected in larger amounts. The filtrates of broth cultures of a number of varieties were tested, but only one was obtained in which poisonous products were abundantly present. Death in guinea-pigs weighing 300 grams followed within fifteen minutes after an injection of 2 c.c.; 1 c.c. had little effect.

As bacteria in milk are swallowed and not injected under the skin, it seemed wise to test the effect of feeding them to very young animals. We therefore fed forty-eight cultures of 139 varieties of bacteria to kittens of two to ten days of age by means of a glass tube. The kittens received 5 to 10 c.c. daily for from three to seven days. Only one culture produced illness or death. Very young guinea-pigs were fed in the same manner with similar results.

After five years of effort to discover some relation between special varieties of bacteria found in milk and the health of children the conclusion has been reached that neither through animal tests nor the isolation from milk given sick infants have we been able to establish such a relation. Pasteurized or "sterilized" milk is rarely kept longer than thirty-six hours, so that varieties of bacteria which after long standing develop in such milk did not enter into our problem. The harmlessness of cultures given to healthy young kittens does not, of course, prove that they would be equally harmless in infants. Even if harmless in robust infants, they might be injurious when summer heat and previous disease had lowered the resistance and the digestive power of the subjects.

**Streptococci in Relation to Disease.**—In an investigation by Dr. H. D. Bergey connection between diarrhea and pus and streptococci was sometimes found.

The results of this investigation appear to warrant the following conclusions:

1. The occurrence of an excessive number of leukocytes in cows' milk is probably always associated with the presence in the udder of some inflammatory reaction brought about by the presence of some of the ordinary pyogenic bacteria, especially of streptococci.
2. When a cow's udder has once become infected with the pyogenic bacteria, the disease tends to persist for a long time, probably extending over several periods of lactation.
3. Lactation has no causative influence *per se* upon the cellular and bacterial content of cows' milk, though it probably tends toward the aggravation of the disease when the udder is once infected.

It is impossible to differentiate in routine milk examinations the pathogenic streptococci of diseased cows from saprophytic varieties.

Thus it happens that a milk which contains great numbers of streptococci may or may not be more dangerous than one which contains an equal number of other apparently less harmful bacteria. The identification of the varieties present requires great care and is only done in the face of feared infection such as an epidemic of septic throats. Those that produce human diseases, except in infants, are probably always from cows in which the udder has been infected from human sources.

**The Deleterious Effect of Live Bacteria in Milk on Infants.**—We tested this ourselves in the following way: During each of the summers of 1902, 1903, and 1904 a special lot of milk was modified for a group of fifty infants, all of whom were under nine months of age, and distributed daily. To one-half a portion of the milk was given raw; to the other half a portion heated at 60° for twenty minutes.

The modified milk was made from a fairly pure milk mixed with ordinary cream. The bacteria contained in the milk numbered on the average 45,000 per cubic centimeter, in the cream 30,000,000. The modified raw milk taken from the bottles in the morning averaged 1,200,000 bacteria per cubic centimeter, or considerably less than the ordinary grocery milk; the pasteurized, about 1000; taken in the late afternoon of the same day they had, respectively, about 20,000,000 and 50,000.

Twenty-one predominant varieties of bacteria were isolated from six specimens of this milk collected on different days. The varieties represented the types of bacteria frequently found in milk. The infants were selected during the first week in June, and at first all were placed on pasteurized milk. The fifty infants which had been selected were now separated into two groups as nearly alike as possible. On the fifteenth of June the milk was distributed without heating to one-half the infants, the other half receiving as before the heated milk. In this way the infants in the two groups received milk of identically the same quality, except for the changes produced by heating to 165° F. for thirty minutes. The infants were observed carefully for three months and medical advice was given when necessary. When severe diarrhea occurred barley water was substituted for milk.

The first season's trial gave the following results: Within one week 20 out of 27 infants put on raw milk suffered from moderate or severe diarrhea; while during the same time only 5 cases of moderate and none of severe diarrhea occurred in those taking pasteurized milk. Within a month 8 of the 27 had to be changed from raw back to heated milk, because of their continued illness; 7, or 25 per cent., did well all summer on raw milk. On the other hand, of those receiving the pasteurized milk, 75 per cent. remained well, or nearly so, all summer, while 25 per cent. had one or more attacks of severe diarrhea. There were no deaths in either group of cases.

During the second summer a similar test was made with 45 infants. Twenty-four were put on raw modified milk; 13 of these had serious diarrhea, in 5 of whom it was so severe that they were put back upon heated milk; 10 took raw milk all summer without bad effects; 2 died, 1 from gross neglect on the part of the mother, the other from d Of the 21 on pasteurized milk, 5 had severe attacks of diarrhea, were kept on this milk except for short periods, when all fo omitted; 16 did well throughout the summer. One infant, m rachitic, died. The third summer's results have not been tabula were similar to those of the first two tests.

The outcome of these observations during the first two s are summarized in the following table:

| Kinds of milk (number of bacteria when consumed). | Number of infants. | Remained well for entire summer. | Number having severe or moderate diarrhea. | Average number days off milk during summer. | Average weekly gain in weight, oz. | Averag numbe of day diarrhe |
|---|---|---|---|---|---|---|
| Pasteurized milk, 1000–50,000 bacteria per c.c. . . . . . | 41 | 31 | 10 | 3.0 | 4.0 | 3.9 |
| Raw milk, 1,200,000–20,000,000 bacteria per c.c. . . . . | 51[1] | 17 | 33 | 5.5 | 3.5 | 11.5 |

Although the number of cases was not large, the results, identical during the three summers, indicate that even a fai milk, when given raw in hot weather, causes illness in a muc percentage of cases than the same milk given after pasteu A considerable percentage of infants, however, do apparently well on raw as on pasteurized milk.

**Bacteria in Milk. Effect on Older Children.**—The childr three years of age who received unheated milk, containing at times from 145,000 to 350,000,000 bacteria per cubic cen showed almost no gastro-intestinal disturbance. The condi three institutions will serve as examples.

In the first of these an average grade of raw milk was use during the summer, contained from 2,000,000 to 30,000,000 per cubic centimeter. This milk was stored in an ice-box until r It was taken by children unheated and yet no case of diarrhe ficient gravity to send for a physician occurred during the entire This institution was an orphan asylum containing 650 children fr to fourteen years of age—viz., three to five years, 98; five to eig 162; eight to fourteen years, 390.

A second institution used an unheated but very pure mil was obtained from its own farm. This milk averaged 50,0 teria per cubic centimeter. The inmates were 70 children ranging from three to fourteen years. In this institution not case of diarrheal disease of any importance occurred during the

In a third institution an average grade of milk was used wh heated. This milk before heating contained 2,000,000 to 20 bacteria per cubic centimeter. This institution was an infant asylum in which there were 126 children between the ages of two and five years. There were no cases of diarrhea during the summer.

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[1] Thirteen of the 51 infants on raw milk were transferred before the end o to pasteurized milk because of serious illness. If these infants had been left on it is believed by the writers that the comparative results would have been unfavorable to raw milk.

These clinical observations taken in connection with the bacteriological examination at the laboratory show that although the milk may come from healthy cattle and clean farms and be kept at a temperature not exceeding 60° F., a very great increase in the number of bacteria may occur. Furthermore, this may occur without the accumulation in the milk of sufficient poisonous products or living bacteria to cause appreciable injury in children over three years of age, even when such milk is consumed in considerable amount and for a period extending over several months. Milk kept at temperatures somewhat above 60° F. was not met with in our investigations, but the histories of epidemics of ptomain poisoning teach that such milk may be very poisonous. It is also to be remembered that milk abounding in bacteria on account of its being carelessly handled is also always liable to contain pathogenic organisms derived from human or animal sources.

**Results with Very Impure Milk Heated vs. Those with Pure or Average Milk Heated.**—During the summer of 1901 we were able to observe a number of babies fed on milk grossly contaminated by bacteria. In 1902 systematic supervision of all stores selling milk was instituted by the Health Department, so that the very worst milk was not offered for sale that summer.

The observations upon the impure milk of 1901 are of sufficient importance to be given in detail, although already mentioned in the report of the observations upon infants of both summers which were fed on "store milk." A group of over 150 infants was so divided that 20 per cent. were allowed to remain on the cheapest store milk which they were taking at the time. To about the same number was given a pure bottled milk. A third group was fed on the same quality of milk as the second, but sterilized and modified at the Good Samaritan Dispensary. A fourth group received milk from an ordinary dairy farm. This milk was sent to a store in cans and called for by the people. A few infants fed on breast and condensed milk were observed for control.

In estimating the significance of the observations recorded in the tables, one should bear in mind that not only do different infants possess different degrees of resistance to disease, but that, try as hard as the physicians could, it was impossible to divide the infants into groups which secured equal care and were subjected to exactly the same conditions. It was necessary to have the different groups in somewhat different parts of the city. It thus happened that the infants on the cheap store milk received less home care than the average, and that those on the pure bottled milk lived in the coolest portion of the city. Certain results were, however, so striking that their interpretation is fairly clear. It is to be noted that the number of infants included in each group is small.

There is nothing in the observations to show that fairly fresh milk from healthy cows, living under good hygienic conditions and containing, on some days, when delivered, as many as 200,000 bacteria per cubic centimeter, had any bacteria or any products due to bacteria that remained deleterious after the milk was heated to near the boiling-point.

TABLE SHOWING THE RESULTS OF FEEDING DURING JULY AND AUGUST, 1901, IN TENEMENT HOUSES, OF 112 BOTTLE-FED INFANTS UNDER ONE YEAR OF AGE, AND OF 47 BOTTLE-FED INFANTS BETWEEN ONE AND TWO YEARS OF AGE WITH MILK FROM DIFFERENT SOURCES, AND THE NUMBER OF BACTERIA PRESENT IN THE MILK.

| Character of milk. | Infants under one year. | | | | | Infants over one year. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Number of infants. | Average weekly gain. | Diarrhea. | | Deaths. | Number of infants. | Average weekly gain. | Diarrhea. | | Deaths. |
| | | | Mild. | Severe. | | | | Mild. | Severe. | |
| 1. Pure milk boiled and modified at dispensary or stations; given out in small bottles; milk before boiling averaged 20,000 bacteria per c.c.; after boiling 2 per c.c. | 41 | oz.<br>3 | 10 | 8 | 1[1] | | oz. | | | |
| 2. Pure milk, twenty-four hours old, sent in quart bottles to tenements, heated and modified at home, 20,000 to 200,000 bacteria per c.c. when delivered. | 23 | 4¾ | 8 | 5 | 0 | 24 | 4½ | 8 | 2 | 0 |
| 3. Ordinary milk, thirty-six hours old, from a selected group of farms, kept cool in cans during transport; 1,000,000 to 25,000,000 bacteria per c.c., heated and modified at home before using. | 18 | 4 | 6 | 6 | 1[2] | 12 | 4 | 1 | 2 | 0 |
| 4. Cheap milk, thirty-six to sixty hours old, from various small stores, derived from various farms, some fairly clean, some very dirty; 400,000 to 175,000,000 bacteria per c.c. before home heating. | 21 | ¼ | 4 | 13 | 4[3] | 7 | ½ | 1 | 3 | 0 |
| 5. Condensed milk of different brands; made up with hot water; as given, contained bacteria from 5000 to 200,000 per c.c. | 9 | ¼ | 5 | 2 | 3 | 4 | 3¾ | 1 | 3 | 0 |
| 6. Breast milk . . . . . . . . | 16 | 2¼ | 5 | 2 | 0 | | | | | |

On the other hand, it is possible that certain varieties of bacteria may, under conditions that are insanitary, find entrance to milk and survive moderate heat or may develop poisonous products resistant to heat in sufficient amount to be harmful, even when they have accumulated to less than 200,000 per c.c.

Turning now to the results of feeding with milk which has been heated and which before sterilization contained from 1,000,000 to 25,000,000 bacteria per cubic centimeter, averaging about 15,000,000, though obtained from healthy cows living under fairly decent conditions and although the milk was kept moderately cool in transit, we find a distinct increase in the amount of diarrheal diseases. Though it is probable that the excessive amount of diarrhea in this group of children was due to bacterial changes which were not neutralized by heat or to living bacteria which were not killed, yet it is only fair to consider that the difference was not very great and that the infants of this group were under surroundings not quite so good as those on the pure milk.

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[1] This infant died from enteritis and toxemia.

[2] This infant died of pneumonia. There had been no severe intestinal disorder noted.

[3] One of the four had pertussis, the remaining three died from uncomplicated enteritis.

Finally, we come in this comparison to the infants who received the cheap store milk after heating. This milk had frequently to be returned because it curdled when boiled, and contained, according to the weather, from 4,000,000 to 200,000,000 bacteria per cubic centimeter. In these infants the worst results were seen. This is shown not only by the death-rate, but by the amount and by the severity of the diarrheal diseases, and the general appearance of the children as noted by the physicians. Although the average number of bacteria in the milk received by this group is higher than that received by the previous group, the difference in results between this group and the previous one can hardly be explained by the difference in the number of bacteria. The varieties of bacteria found in this milk were more numerous than in the better milk, but we were unable to prove that they were more dangerous. Probably the higher temperature at which the milk was kept in transit, and the longer interval between milking and its use, allowed more toxic bacterial products to accumulate.

**Bacterial Contamination of Milk—General Conclusions[1] as to Relative Importance.**—1. During cool weather neither the mortality nor the health of the infants observed in the investigation was appreciably affected by quality of the market milk or by the number of bacteria which it contained. The different grades of milk varied much less in the amount of bacterial contamination in winter than in summer, the store milk averaging only about 750,000 bacteria per cubic centimeter.

2. During hot weather, when the resistance of the children was lowered the kind of milk taken influenced both the amount of illness and the mortality; those who took condensed milk and cheap store milk did the worst, and those who received breast milk, pure bottled milk, and modified milk did the best. The effect of bacterial contamination was very marked when the milk was taken without previous heating; but, unless the contamination was very excessive, only slight when heating was employed shortly before feeding.

3. The number of bacteria which may accumulate before milk becomes noticeably harmful to the average infant in summer differs with the nature of the bacteria present, the age of the milk, and the

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[1] These conclusions were drawn up by the authors in association with Dr. L. E. Holt, after a joint study of the results obtained in the studies above recorded.

temperature at which it has been kept. When the milk is taken raw, the fewer the bacteria present the better are the results. Of the usual varieties, over 1,000,000 bacteria per cubic centimeter are certainly deleterious to the average infant. However, many infants take milk without apparently harmful results. Heat of 145° F. for thirty minutes or of 170° F. for a shorter period not only destroys most of the bacteria present, but, apparently, some of their poisonous products. No harm from the bacteria previously existing in recently heated milk was noticed in these observations unless they had amounted to many millions, but in such numbers they were decidedly deleterious.

4. When milk of average quality was fed, pasteurized and raw, those infants who received milk previously heated did, on the average, much better in warm weather than those who received it raw. The difference was so quickly manifest and so marked that there could be no mistaking the meaning of the results.

5. No special varieties of bacteria were found in unheated milk which seemed to have any special importance, in relation to the summer diarrheas of children. A few cases of acute indigestion were seen immediately following the use of pasteurized milk more than thirty-six hours old. Samples of such milk were found to contain more than 100,000,000 bacteria per cubic centimeter, mostly spore-bearing varieties. The deleterious effects, though striking, were neither serious nor lasting.

6. After the first twelve months of life infants are less and less affected by the bacteria in milk derived from healthy cattle and the air. According to these observations, when the milk had been kept cool, the bacteria did not appear to injure the children over three years of age at any season of the year, unless in very great excess.

7. While it is true that even in tenements the results with the best bottle feeding are nearly as good as average breast feeding, it is also true that most of the bottle feeding is at present very badly done; so that, as a rule, the immense superiority of breast feeding obtains. This should therefore be encouraged by every means, and not discontinued without good and sufficient reasons. The time and money required for artificial feeding, if expended by the tenement mother to secure better food and more rest for herself, would often enable her to continue nursing with advantage to her child.

**Influence of Temperature upon the Multiplication of Bacteria in Milk.**—Few, even of the well informed, appreciate how great a difference a few degrees of temperature will make in the rate of bacterial multiplication. Milk rapidly and sufficiently cooled keeps almost unaltered for thirty-six hours, while milk insufficiently cooled deteriorates rapidly.

The majority of the bacteria found in milk grow best at temperatures above 70° F., but they also multiply slowly even at 40° F.; thus, of 60 species isolated by us, 42 developed good growth at the end of seven days at 39° F. Our observations have shown that the bacteria slowly increase in numbers after the germicidal properties of the milk have disappeared, and the germs have become accustomed to the low temperature. In fact, milk cannot be permanently preserved unaltered unless kept at 32° F. or less. The degree of cooling to which ordinary supplies of milk are subjected differs greatly in various localities. Some farmers chill their milk rapidly, by means of pipe coils over which the milk flows; others use deep wooden tanks filled with water into which the cans of milk are placed soon after milking. In winter these methods are very satisfactory, for the water runs into the pipes or tanks at about 38° F. In warmer weather they are unsatisfactory, unless ice is used, as the natural temperature of the water may be as high as 55° F. A considerable quantity of milk is not cooled at all at the farms. It is sent to the creamery or railroad after two to six hours, and is then more or less cooled. These few hours in summer, when the milk is left almost at blood heat, allow an enormous development of bacteria to take place, as is shown in the table below.

TABLE I.—SHOWING THE DEVELOPMENT OF BACTERIA IN TWO SAMPLES OF MILK MAINTAINED AT DIFFERENT TEMPERATURES FOR TWENTY-FOUR, FORTY-EIGHT, AND NINETY-SIX HOURS, RESPECTIVELY. THE FIRST SAMPLE OF MILK WAS OBTAINED UNDER THE BEST CONDITIONS POSSIBLE, THE SECOND IN THE USUAL WAY. WHEN RECEIVED SPECIMEN, NO. 1 CONTAINED 3000 BACTERIA PER C.C., SPECIMEN NO. 2, 30,000 PER C.C.

| Temperature. Fahrenheit. | Time which elapsed before making tests. | | | |
|---|---|---|---|---|
| | 24 hours. | 48 hours. | 96 hours. | 168 hours. |
| 32° | 2,400 | 2,100 | 1,850 | 1,400 |
| | **30,000**[1] | **27,000** | **24,000** | **19,000** |
| 39° | 2,500 | 3,600 | 218,000 | 4,209,000 |
| | **38,000** | **56,000** | **4,300,000** | **38,000,000** |
| 42° | 2,600 | 3,600 | 500,000 | 11,200,000 |
| | **43,000** | **210,000** | **5,760,000** | **120,000,000** |
| 46° | 3,100 | 12,000 | 1,480,000 | 80,000,000 |
| | **42,000** | **360,000** | **12,200,000** | **300,000,000** |
| 50° | 11,600 | 540,000 | 300,000,000 | 1,000,000,000[2] |
| | **89,000** | **1,940,000** | **1,000,000,000**[2] | |
| 55° | 18,800 | 3,400,000 | | |
| | **187,000** | **38,000,000** | | |
| 60° | 180,000 | 28,000,000 | | |
| | **900,000** | **168,000,000** | | |
| 68° | 450,000 | 500,000,000 | | |
| | **4,000,000** | **1,000,000,000**[2] | | |

OBSERVATIONS ON BACTERIAL MULTIPLICATION IN MILK AT 90° F., A TEMPERATURE COMMON IN NEW YORK IN HOT SUMMER WEATHER.

TABLE II.—NUMBER OF BACTERIA PER C.C.

| | Milk I. Fresh and of good quality. | Milk II. Fair quality from store. | Milk III. Bad quality from store. |
|---|---|---|---|
| Original number . . . . . | 5,200 | 92,000 | 2,600,000 |
| After two hours . . . . . | 8,400 | 184,000 | 4,220,000 |
| After four hours . . . . . | 12,400 | 470,000 | 19,000,000 |
| After six hours . . . . . | 68,500 | 1,260,000 | 39,000,000 |
| After eight hours . . . . | 654,000 | 6,800,000 | 124,000,000 |

A sample of milk No. 1 removed after six hours and cooled to 50° F. contained 145,000,000 at the end of twenty-four hours. Some of this milk, kept cool from the beginning contained but 12,800 bacteria per cubic centimeter at the end of twenty-four hours.

[1] The figures referring to tests of the second sample are printed in heavy-face type.

[2] These figures signify the maximum growth and are conservative estimates only.

**Pasteurization of Milk.**—The two dominant factors which control the temperature and time at which the milk should be heated are (1) the thermal death-points of pathogenic bacteria, and (2) the thermolabile food constituents of the milk. The first factor is almost equally important for milk used by persons of all ages, while the second factor is only important for milk used for very young children.

The exposure of bacteria for a short time at a high temperature is equivalent to a longer time at a lower temperature. The ferments and other labile food constituents, on the other hand, are altered much more by the higher temperature. It is well, therefore, to choose the lowest possible temperature which will kill the non-sporebearing pathogenic bacteria in a practicable length of time. Such an exposure is 60° C. (140° F.) for thirty minutes, 70° C. (158° F.) for five minutes. Very much shorter exposures, as one minute at 70° C., will kill the great majority of pathogenic and other bacteria in the milk and add much of safety, as seen in the tables below, but it is better to be on the safe side.

TABLE SHOWING EFFECT OF HEAT UPON TUBERCLE BACILLI IN MILK.

| Degree of heat. | Time exposed. | Amount of milk. | Result in guinea-pigs. |
|---|---|---|---|
| 60° C. | 15.0 min. | 1 c.c. | Infection. |
| 60° C. | 20.0 min. | 1 c.c. | No infection. |
| 60° C. | 30.0 min. | 1 c.c. | No infection. |
| 70° C. | 0.5 min. | 1 c.c. | Infection.[1] |
| 70° C. | 1.0 min. | 1 c.c. | No infection. |
| 70° C. | 2.0 min. | 1 c.c. | No infection. |
| Control not heated | | 0.001 | Infection. |

This milk was infected by adding one-fifth of its quantity of sputum rich in tubercle bacilli.

**Development of Bacteria in Milk which had been Heated.**—There is a common idea that bacteria develop much more rapidly in milk that has been heated than in raw milk. This is only true for freshly drawn milk which has slight bactericidal power.

The table below shows the effect on bacteria in milk of heating to 70° C. for one-half and one minute. Not only the immediate reduction in number is seen to be great, but the difference continues when the milk is kept cold for two days.

TWO SAMPLES MIXED FROM 100 SAMPLES FROM INSPECTORS.
PASTEURIZED AT 160° F. PLATES MADE SAME DAY.

| Sample I. | | Sample II. | |
|---|---|---|---|
| Raw milk | 600,000 | Raw milk | 5,400,000 |
| ½ minute pasteurized | 2,000 | ½ minute pasteurized | 7,400 |
| 1 minute pasteurized | 1,000 | 1 minute pasteurized | 600 |
| SAME SAMPLES KEPT IN ICE-BOX TWENTY-FOUR HOURS AT 45° F. (7° C.). | | | |
| Raw milk | 6,300,000 | Raw milk | 21,600,000 |
| ½ minute pasteurized | 18,000 | ½ minute pasteurized | 12,000 |
| 1 minute pasteurized | 900 | 1 minute pasteurized | 3,600 |
| IN ICE-BOX FORTY-EIGHT HOURS AT 45° F. (7° C.). | | | |
| Raw milk | 16,200,000 | Raw milk | 63,000,000 |
| ½ minute pasteurized | 120,000 | ½ minute pasteurized | 276,000 |
| 1 minute pasteurized | 10,000 | 1 minute pasteurized | 90,000 |
| IN ROOM AT 71° F. (22° C.). | | | |
| Raw milk | 36,600,000 | Raw milk | 150,000,000 |
| ½ minute pasteurized | 5,400,000 | ½ minute pasteurized | 4,500,000 |
| 1 minute pasteurized | 5,400,000 | 1 minute pasteurized | 3,600,000 |

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[1] Most of the guinea-pigs were not infected by the milk heated for one-half minute.

NUMBER OF BACTERIA IN MILK PRODUCED UNDER DIFFERENT CONDITIONS.

1. The number of bacteria present at the time of milking and twenty-four, forty-eight, and seventy-two hours afterward in milk obtained and kept under correct conditions.

No preservatives were present in any of the following specimens:

Pure milk obtained where every reasonable means was taken to ensure cleanliness. The long hairs on the udder were clipped; the cows roughly cleaned and placed in clean barns before milking; the udders were wiped off just previous to milking; the hands of the men were washed and dried; the pails used had small (six-inch) openings, and were thoroughly cleaned and sterilized by steam before use. Milk cooled within one hour after milking to 45° F., and subsequently kept at that temperature. The first six spec mens were obtained from individual cows; the last six from mixed milk as it flowed at different times from the cooler. Temperature of barns 55° F.

NUMBER OF BACTERIA IN 1 C.C. OF MILK.

| | 5 hours after milking. | After 24 hours, | After 48 hours. | After 72 hours. |
|---|---|---|---|---|
| FROM SIX INDIVIDUAL COWS. | | | | |
| | 500 | 700 | 12,500 | Not counted. |
| | 700 | 700 | 29,400 | Not counted. |
| | 19,900 | 5,200 | 24,200 | Not counted. |
| | 400 | 200 | 8,600 | Not counted. |
| | 900 | 1,600 | 12,700 | Not counted. |
| | 13,000 | 3,200 | 19,500 | Not counted. |
| Average | 6,000 | 1,933 | 17,816 | |
| FROM SAMPLES OF MIXED MILK OF ENTIRE HERD. | | | | |
| | 6,900 | 12,000 | 19,800 | 494,000 |
| | 6,100 | 2,200 | 20,200 | 550,000 |
| | 4,100 | 700 | 7,900 | 361,000 |
| | 1,200 | 400 | 7,100 | 355,000 |
| | 6,000 | 900 | 9,800 | 445,000 |
| | 1,700 | 400 | 8,700 | 389,000 |
| Average | 4,333 | 2,766 | 10,583 | 329,000 |

Twenty-five samples taken separately from individual cows on another day and tested immediately averaged 4550 bacteria per cubic centimeter and 4500 after twenty-four hours. These twenty-five specimens were kept at between 45° and 50° F.

2. Milk taken during winter in well-ventilated, fairly clean, but dusty barns. Visible dirt was cleaned off the hair about the udder before milking. Milkers' hands were wiped off, but not washed. Milk pails and cans were clean, but the straining cloths dusty. Milk cooled within two hours after milking to 45° F.

NUMBER OF BACTERIA IN 1 C.C. OF MILK.

| | At time of milking. | After 24 hours. | After 48 hours. |
|---|---|---|---|
| | 12,000 | 14,000 | 57,000 |
| | 13,000 | 20,000 | 65,000 |
| | 21,500 | 31,000 | 106,000 |
| Average | 15,500 | 21,666 | 76,000 |

NUMBER IN CITY MILK.

3. The condition of the average raw city milk is very different, and is shown in the following tables.

The twelve samples were taken late in March, 1912, by Inspectors of the Department of Health of New York City from cans of milk immediately upon their arrival in the city. Raw milk at present gives similar counts.

The temperature of the atmosphere averaged 50° F. during the previous twenty-four hours. The temperature of the milk when taken from the cans averaged 45° F. Much of this milk had been carried over two hundred miles. From the time of its removal from the cans, which was about 2 A.M., until its distribution in nutrient agar, at 10 A.M., the milk was kept at about 45° F.

| FROM NEW YORK AND HUDSON RIVER RAILROAD. | | FROM HARLEM RAILROAD. | |
|---|---|---|---|
| No. of sample. | No. of bacteria in 1 c.c. | No. of sample. | No. of bacteria in 1 c.c. |
| 50 | 35,200,000 | 48 | 6,200,000 |
| 51 | 13,000,000 | 49 | 2,200,000 |
| 52 | 2,500,000 | 50 | 15,000,000 |
| 53 | 1,400,000 | 51 | 70,000 |
| 54 | 200,000 | 52 | 80,000 |
| 55 | 600,000 | 53 | 320,000 |

The results of the examination of many thousands of specimens last year indicated that most of the milk of grade A was of fair quality, but that the raw milk of the other grades contained excessive numbers of bacteria before pasteurization.

It must be kept in mind that milk averaging 3,000,000 bacteria per cubic centimeter will, when kept at the temperature common in the homes of the poor who comprise the larger part of the population, soon contain very largely increased numbers and show its dangerous condition by turning sour and curdling.

**Cleanliness Used in Obtaining Milk, and its Influence.**—The present conditions under which much of the milk is obtained are not pleasant to consider. In winter, and to a less extent at other seasons of the year, the cows in many stables stand or lie down in stalls in the rear portion of which there is altogether too much manure and urine. When milked the hands of the milkers are not cleansed, nor are the under portions of the cows, only visible masses of manure adhering to the hair about the udder being removed. Some milkers even moisten their hands with milk, to lessen friction, and thus wash off the dirt of their hands and the cow's teats into the milk in the pails. Some may regard it as an unnecessary refinement to ask that farmers should thoroughly clean the floors of their stalls once each day, that no sweeping should be done just before milking, and that the udders should be wiped with a clean damp cloth and the milkers should thoroughly wash and wipe their hands before commencing milking. The pails and cans should not only be carefully cleansed, but afterward scalded out with boiling water. The washing of the hands would lessen the number of ordinary filth bacteria in the milk, and diminish risk of transmitting to milk human infectious diseases, like scarlet fever, diphtheria, and enteric fever, by the direct washing off of the disease germs from infected hands. It would also inculcate general ideas of the necessity of cleanliness and of the danger of transmitting disease through milk. The value of cleanliness in limiting the number of bacteria is demonstrated by the figures contained in the tables.

**General Conclusions.**—Because of its location and its hairy covering, the cow's udder is always more or less soiled with dirt and manure unless cleaned. On account of the position of the pail and the access of dust-laden air it is impossible to obtain milk by the usual methods without mingling with it a considerable number of bacteria. With suitable cleanliness, however, the number is far less than when filthy methods are used, there being no reason why fresh milk should contain in each cubic centimeter, on the average, more than 12,000 bacteria per cubic centimeter in warm weather and 5000 in cold weather. Such milk, if quickly cooled to 46° F., and kept at that temperature, will at the end of thirty-six hours contain on the average less than 50,000 bacteria per cubic centimeter, and if cooled to 40° F. will average less than its original number.

With only moderate cleanliness such as can be employed by anyone without adding appreciably to his expense, namely, clean pails, with small openings, straining cloths, cans or bottles, and hands, a clean place for milking, and a decent condition of the cow's udder and belly, milk when first drawn will not average in hot weather over 30,000, and in cold weather not over 25,000 bacteria per cubic centimeter. Such milk, if cooled and kept at 50° F., will not contain at the end of twenty-four hours over 100,000 bacteria per cubic centimeter. If kept at 40° F. the number of bacteria will not be over 100,000 per cubic centimeter after forty-eight hours.

If, however, the hands, cattle, and barns are filthy and the pails are not clean, the milk obtained under these conditions will, when taken from the pail, contain very large numbers of bacteria, even up to 1,000,000 or more per cubic centimeter.

Freshly drawn milk contains a slight and variable amount of bactericidal substances which are capable of inhibiting bacterial growth. At temperatures under 50° F. these substances act efficiently (unless the milk is filthy) for from twelve to twenty-four hours, but at higher temperatures their effect is very soon completely exhausted, and the bacteria in such milk will then rapidly increase. Thus the bacteria in fresh milk which originally numbered 5000 per cubic centimeter decreased to 2400 in the portion kept at 42° F. for twenty-four hours, but rose to 7000 in that kept at 50° F., to 280,000 in that kept at 65° F., and to 12,500,000,000 in the portion kept at 95° F.

As we have seen, the milk in New York City before general pasteurization was adopted was found on bacteriological examination to contain, as a rule, excessive numbers of bacteria. During the cold weather the raw milk in the shops averaged over 300,000 bacteria per cubic centimeter, during cool weather about 1,000,000, and during hot weather about 2,000,000.

The above statement holds for milk sold at the ordinary shops, and not that of the best of the special dairies, where, as previously stated, the milk contained only from 1000 to 30,000 bacteria, according to the season of the year. The committee on the bacteriological examination of milk of the A. P. H. A. at the meeting at Cincinnati in

October, 1916, presented the following statement of the interpretation to be placed on milk counts and the number of bacteria permissible.

1. Where the analysis can be made immediately after the milking the number of bacteria enables conclusions to be drawn as to the cleanliness and care in the dairy and the thoroughness in the cleaning and sterilization of the milk vessels, or sometimes the presence of cows with diseased udders. With properly cleaned and sterilized milk vessels and proper care in the farm and dairy the numbers of bacteria should not exceed 10,000, and may be easily brought down to 5000. Numbers beyond these in milk analyzed immediately after the milking may be regarded as an indication of unclean dairy methods, dirty and unsterile milking vessels, or to diseased udders. Apart from diseased udders the factors in dairying that most noticeably increase the bacteria count are unclean udders, milking with wet hands, unsterile milk vessels, unsterile strainers, and failure to cool the milk promptly.

2. If the milk is properly cooled with ice the numbers should not materially increase in five to seven hours. Communities within five to seven hours of their dairies should be able therefore to obtain milk with nearly as low a count as above indicated. Hence in such communities bacterial counts above these numbers should not be found in properly guarded milk. A count of 50,000 in such a community is an indication either of unsatisfactory dairy conditions or of failure to properly cool the milk during transportation. Night's milk if properly cooled can also easily be brought within these limits if analyzed the next morning. A count of over 50,000 for a community close to the dairies must be regarded as unsatisfactory, and the number should not be much more than 10,000 for high grade milk. In hot summer weather the difficulties of keeping low counts are greater, but even then they need not surpass 30,000 if the milk is properly cooled.

3. Where milk must be a longer time in transportation from the dairy there will be inevitably an increase in bacteria, depending on the length of time and the temperature. Experience has shown, however, that even in these conditions the excessively high numbers that have frequently been found in city milk are in reality due to dirty dairy conditions, to dirty and unsterile milk utensils, or culpable neglect in cooling. Moreover, such high bacterial counts at the shipping station are frequently traceable to a few dirty dairies whose milk with an abnormally high count contaminates the rest of the supply. Dirty shipping cases and warm temperatures in shipping are responsible for most of the high bacterial counts in city milk. Where the milk from healthy cows reaches the city within twenty-four hours, however, the number should not be over 100,000 in winter or 200,000 in summer, and numbers in excess of this may be regarded as due either to improper dairy conditions, dirty milk vessels, or insufficient cooling. In larger cities where much of the milk is forty-eight hours in reaching the city higher numbers may naturally be expected. But even under these conditions there is no good reason why the number of bacteria should reach 1,000,000; and it may mostly be brought down to below 200,000.

In such cities, therefore, milk with more than 1,000,000 bacteria must be regarded as improperly guarded either at the dairy or on its transit.

4. For a Grade A milk higher demands should be made than for the ordinary grade. The standard set by the Milk Commission for Grade A, viz., of 200,000 for milk to be subsequently pasteurized or for 100,000 to be used raw, is stated by that Commission to be an extreme limit for the most unfavorable conditions. Cities situated near the supplying dairies should demand a much higher standard, which should not allow over 10,000 in bacterial content in Grade A milk in communities favorably situated.

5. For communities situated where ice is not available it may be necessary to accept a milk with a higher bacterial content; but as rapidly as possible the standard should be made to approach the limits above given.

The question might be raised, Are even these enormous numbers of bacteria often found in milk during hot weather harmful?

Our knowledge is probably as yet insufficient to state just how many bacteria must accumulate to make them noticeably dangerous in milk. Some varieties are undoubtedly more harmful than others, and we have no way of restricting the kinds that will fall into milk, except by enforcing cleanliness. We have also to consider that milk is not entirely used for some twelve hours after being purchased, and that during all this time bacteria are rapidly multiplying, especially where, as among the poor, no provision for cooling it is made. Slight changes in the milk which to one child would be harmless, would in another produce disturbances which might lead to serious disease. A safe conclusion is that no more bacterial contamination should be allowed than it is practicable to avoid. Any intelligent farmer can use sufficient cleanliness and apply sufficient cold, with almost no increase in expense, ½ cent per quart, to supply milk twenty-four to thirty-six hours old which will not contain in each cubic centimeter over 50,000 to 100,000 bacteria, and no milk containing more bacteria should be sold.

The most deleterious changes which occur in milk during its transportation are now known not to be due to skimming off the cream or to the addition of water, but to the changes produced in the milk by multiplication of bacteria. During this multiplication, acids and distinctly poisonous bacterial products are added to the milk, to such an extent that much of the milk has become distinctly deleterious to infants and invalids. It is the duty of health authorities to prevent the sale of milk rendered unfit for use because of excessive numbers of bacteria and their products.

The culture tests to determine the number of bacteria present in any sample of milk require at least forty-eight hours; so that the sale of milk found impure cannot be prevented. It will, however, be the purpose of the authorities gradually to force the farmers and the middlemen to use cleanliness, cold, and dispatch in the handling of their milk, rather than to prevent the use of the small amount tested on any one day.

If the milk on the train or at the dealer's w
excessive numbers of bacteria, the farmers wo
instructed to carry out the simple necessary rule
printed form.

**Transmission of Contagious Diseases throu
Bacteria in Milk.**—Tuberculosis, typhoid feve
scarlet fever, and diphtheria are the chief dis
means of milk in this locality. In other countries
and possibly other diseases may be due at tim
The obscure disease *trembles* is also believed to be

The tubercle bacilli are in the majority of cases
but may come from human sources, the typhoid b
man, the contagion of true scarlet fever convey
always from man, but the contagion of a disease
certainly given off by cows suffering from certain
not fully identified. Diphtheria bacilli are proba
origin, as animals, except cats, practically never s
and these only under exceptional conditions. Th
septic throats are probably usually from human
mation but may at times come from septic cows
kept below 60° F. the typhoid bacilli and the str
pathogenic germs that we believe increase in any

The following epidemics and cases have been re
of the Marine Hospital Service, as produced by c

Typhoid fever . . . . . . . . . . . . . .
Scarlet fever . . . . . . . . . . . . . .
Sore-throat . . . . . . . . . . . . . .
Diphtheria . . . . . . . . . . . . . .
Tuberculosis

No case of measles, smallpox, chicken-pox, whoo
poliomyelitis has been clearly traced to milk.

**The Relation of the Typhoid Carrier to Milk Infecti**
of typhoid fever have until recently puzzled i
though evidently milk-borne, yet no case of ty
found. The discovery that about 2 per cent. of tho
from typhoid fever remain infected and continue d
lives to pass typhoid bacilli has cleared up the
due to these carriers have already been traced bo
and elsewhere. Many observers have already dis
typhoid cases to milk infection. Hands, water, fl
in the transfer of the bacilli from the dejecta to
we traced over 400 cases to infection of a milk
carrier who had the disease forty-seven years ag

**The Conveyance of Scarlet Fever by Means of**
know the organism which excites scarlet fever, we
the means by which it is spread as we are in the
typhoid fever, and diphtheria. We know, howe

secretions are dangerous. Where the infection has been traced it has usually been found that the milker has suffered from an unrecognized case or is convalescent. It seems as if the contagion must either increase in milk or be capable of infecting when greatly diluted, for cases have developed from milk after great dilution. A small number of epidemics have appeared to come from the milk of diseased cows. Many are skeptical about this, but after personal experience we think it probable. The history of one case was as follows: The milk from a septic cow was delivered to two schools. About thirty of the boys who drank the milk developed the disease while none of the day scholars who went home to lunch did. Some of the cases developed at first only sore-throats, others only the rash. On the second day the cases resembled very closely scarlet fever. There was no scarlet fever in the town. The milk contained immense numbers of long-chained streptococci.

Diphtheria and septic sore-throats are occasionally produced by milk. The diphtheria bacilli usually originate from a mild case, the nature of which is not detected. Septic sore-throats produced by milk are usually traced to contamination from a human source, but like cases developing the scarlet rash the infection may come from cows suffering from some acute septic udder disease.

**The Grading of Milk.**—The appreciation of the importance of the bacteria in milk has led to the pasteurization of all milk entering New York City, except that produced and transported under the very best conditions. Together with the farm conditions the bacterial content of milk is used to grade it. All milk in New York City is divided into grades A, B, and C.

Grade A is raw and pasteurized. The raw is from cows which have successfully passed the tuberculin test.

Grade B is all pasteurized.

Grade C is all pasteurized.

The grade A may contain 60,000 bacteria per cubic centimeter.

The grade B may contain 1,500,000 when raw and 50,000 after pasteurization.

The grade C may contain any number within reason before and 100,000 after pasteurization. It is supposed to be used for cooking purposes only. The State of New York has adopted a similar grading except that it allows raw and pasteurized in all three grades. Many cities and States are adopting such grades. A powerful influence in this direction was the report of the national committee of experts on milk standards. This was a commission appointed by the New York Milk Committee.

# CHAPTER L.

## THE BACTERIOLOGICAL EXAMINATION

Of the shellfish commonly used as food, oyster
sively eaten. According to the United States D
of Fisheries (1913): "Economically, oysters are tl
all cultivated water products."[1] He estimates tl
of the world as over 42,000,000 bushels, represent
$25,000,000. The share of the United States in t
88 per cent. of the quantity and about 70 per cent.
fourths of this is controlled by the following States:
Connecticut, Massachusetts, Maryland, New Jers
Louisiana.

In their normal habitat, in sea waters free fro
are free from dangerous bacteria, but since cities a
or near the sea coast find it convenient and advant
water for the disposal of their sewage, oyster-bree
subject to pollution and the oysters infected w
intestinal type. Only when the pollution is sufficie
contamination of the beds avoided and this can o
careful sanitary and bacteriological examinations.
oysters should not be marketed or harvested fro
been recently exposed to dangerous sewage pollu

Serious epidemics of typhoid due to the eatin
have been reported at various times. One of the
called attention to this danger was reported b
Wesleyan University. Investigations showed that
fattened at the mouth of a stream, that a house r
cases of typhoid fever and that drainage from tl
stream.

The period of infection of oysters is transitory,
removed from polluted waters, they will cleanse tl
for food in from six to eight days. This is done at
Sea water is raised mechanically, is passed throu
through sand and finally into reservoirs, containi
the water is slowly changed and the purification

Artificial purification of oysters from polluted b
by Wells,[2] and the method has been tried out on an
harbor at New Haven, Conn. The oysters are
stationary floats containing sea water. Hypochl
in about the proportion of one part of available ch

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[1] Smith, H. M.: National Geographic Magazine, Ma

[2] Public Health Rep., July 14, 1916, pp. 1848, 1852,

thousand parts of water. The water is treated a second time with the hypochlorite, about six hours later. This is to insure the disinfection of organisms which may have escaped the first treatment, by reason of the closing of the oysters when the disinfectant was added until such time as the water is no longer objectionable to them. In twenty-four hours, the total number of organisms in the oysters is greatly reduced and there are practically no bacteria of the B. coli group.

It has been noticed by several observers that there is a seasonal variation in the bacterial contents of oysters. Gorham[1] (1912) in a series of examinations, found that results obtained from oysters examined in the summer did not agree with those obtained from oysters from the same beds examined in the winter. He concluded, therefore, that oysters hibernate.

Experiments carried on in the Research Laboratory of the New York City Health Department (1912) showed that oysters placed in typhoid-infected sea water did not become infected with typhoid bacilli while the temperature was maintained at 3° C. The surrounding water contained 13,000 typhoid bacilli per cubic centimeter. Further experiments along the same lines were made by Pease.[2] He placed oysters and sea water in separate containers in the ice-box and held them at a temperature of 36° F. overnight. Then fuchsin was added to the water and the oysters were placed in the solution. The whole was left in the ice-box all day. Another lot of oysters was placed in fuchsin sea water and kept at a temperature of 65° F. Both sets of oysters were washed in salt water to remove the fuchsin from the shells. When opened the oysters which had been kept at 34° F. showed no trace of fuchsin, while the gills of the oysters kept at 65° F. were turned a dark fuchsin color.

From these experiments he concluded that oysters kept at a temperature of 34° to 36° F. will remain closed, and that since particles of soluble dyes in aqueous solution are much smaller than the bodies of bacteria, bacteria in the waters surrounding the oysters are totally excluded. Furthermore, he says, "Those organisms which have previously gained access to the oyster are destroyed or gradually eliminated so that the total number of bacteria in the oysters is greatly reduced and the oysters become practically free from colon bacilli. Oysters gathered during the hibernating season are more easily handled than during the early part of the oyster season."

**Standard Methods for the Examination of Shellfish Adopted by the American Public Health Association. Oysters in the Shell.**—*Selection of Sample.*—Twelve oysters of the average sizes of the lot under examination, with deep bowels, short lips and shells tightly closed, shall be picked out by hand and prepared for transportation to the laboratory. As complete a record of such data as is possible to obtain shall be made covering the following points: The exact location of the bed from which the sample has been selected. The depth of the water over the bed at time of collection. The state of the tide. The direction and velocity of the wind. Other weather conditions. The day and hour of the removal of the stock from the water. The conditions under which the stock has been kept since removal from the water and prior to the of the sample. The day and the hour of the taking of the sample.

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[1] Am. Jour. Public Health, 1912, ii, 24.

[2] Ibid., November, 1912, ii, 849.

*Transportation of the Sample.*—The oysters so selected shall be pa suitable metal or pasteboard containers of such size and shape that a of them can be enclosed in a shipping case capable of satisfactory refrig by means of ice. The important points in this connection are:

A. The prevention of the mixing of the oyster liquor of different s and of the mixing of the ice-water with the oysters.

B. The icing of the samples if they are not to arrive at the point of lab examinations inside of thirty-six hours or if the outside temperature is 50° F.

It is not necessary to enclose the oysters in an absolutely tight co providing the above conditions are maintained.

*Condition of Samples.*—Record shall be made of the general condition oysters when received, especially whether the shells are open or closed; presence of abnormal odors and of the temperature of the stock.

*Technical Procedure.*—The bacteriological examination shall be sta soon as possible after the receipt of the sample.

The oysters shall be thoroughly cleaned with a stiff brush and clear r water and then dried. The edges of the shell shall be passed through th or burned with alcohol.

The opening of the shell shall be accomplished by either of the fo methods:

A. By the use of a sterile oyster knife in the usual manner.

B. By drilling through a flamed portion of the shell near the hinge sterile drill. The drill shall be sterilized and the site of the operation in t be flamed at least once during the drilling process.

*Bacterial Counts.*—Bacterial counts shall be made of the composite of each lot obtained by mixing the shell liquor of five oysters. Agar s used for the culture medium and in general the procedure shall be in acc with the method recommended for examination of water by the Comm Standard Methods of Water Analysis of the American Public Health Asso

The water used for dilution purposes shall contain 1 per cent. sodium c in order to approximate the natural salinity of oyster liquor.

The agar plates shall be incubated at 20° C. for three days and the c then counted.

A satisfactory method of opening an oyster is to strike it a blow with a hammer directly on the large muscle which holds the together. This injures the oyster just enough to cause the sh spring apart. The edges of the shell are passed through the flan the liquor is poured into a sterile test-tube.

**Determination of Bacteria of the Bacillus Coli Group.**—The quant determination of the presence of B. coli shall be in accordance with the fo procedure:

Measured quantities (1.0, 0.1, 0.01 c.c., etc., or their equivalents in dil of the shell water of each of five oysters selected from the dozen, shall be in fermentation tubes containing lactose peptone bile, prepared accor the method recommended by the Committee on Standard Methods of Analysis. These shall be incubated for three days at 37° C., and the p or absence of gas noted daily. For all ordinary purposes of routine v development of 10 to 85 per cent. of gas during this time period shall con a positive test indicating a presumption of the presence of at least one bac of the Bacillus coli group in the quantity of shell water tested. But r B. coli rating based on these results shall be used for official[1] approval demnation unless positive confirmatory tests for the presence of organisms of the B. coli group shall have been obtained from the tube of highest or next highest dilution from each oyster showing the presence of gas. These confirmatory tests shall be begun immediately upon noting the formation of gas, and carried out in accordance with the procedure recommended by the Committee on Standard Methods of Water Analysis.

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[1] There seems to be no special object gained in confirming the presumptive oysters showing low scores. A low score is approved anyway and confirmation o will not raise the score.

**Statement of Results.**—The results of the bacterial counts shall be expressed as number of bacteria per cubic centimeter. The results of the lists for B. coli shall be expressed either in the form of the following arbitrary numerical system to be known as "The American Public Health Association Method of Rating Oysters for B. coli; or in Estimated Number of Bacteria of the B. coli Group per Cubic Centimeter of the Sample."

The presence of B. coli in each oyster of the five examined is to be given the following values, which represent the reciprocals of the greatest dilutions in which the test for B. coli is positive:

If present in 1 c.c. but not in 0.1 c.c., the value of 1. If present in 0.1 c.c. but not in 0.01 c.c., the value of 10. If present in 0.01 c.c., but not in 0.001 c.c. the value of 100, etc.

The addition of these values for the five oysters would give the total numerical value for the sample, and this figure would be the score for B. coli.

The results should be expressed in the following tabular form:

RESULTS OF TESTS FOR B. COLI IN DILUTIONS INDICATED.

| Oysters. | 1 c.c. | 0.1 c.c. | 0.01 c.c. | Numerical value. |
|---|---|---|---|---|
| 1 | + | + | 0 | 10 |
| 2 | + | + | 0 | 10 |
| 3 | + | 0 | 0 | 1 |
| 4 | + | 0 | 0 | 1 |
| 5 | + | 0 | 0 | 1 |
| | | | Total or score for B. coli = | 23 |

+ = Presence of B. coli group in fermentation tube test with lactose bile where subsequent isolation tests have confirmed the results of the presumptive test or other satisfactory test.

0 = Failure to demonstrate presence of B. coli group.

*Estimated Number of B. Coli per Cubic Centimeter.*[1]—It will be seen that if the B. coli score is divided by 5, the standard number of oysters tested, the results will approximate the number of B. coli per cubic centimeter of shell water. Partly because it does not do this exactly but also for simplicity and the avoidance of fractions, the method of stating results as an arbitrary "score" is preferred by the committee. Practical experience with the method has also appeared to justify this conclusion.

Sometimes results similar to the following are obtained, that is, one or more oysters may show positive results in small quantities of shell water while an equal number may show negative results in larger quantities. In this case the next lower numerical value should be given to the positive results in the high dilutions and such positive results should be considered as being transferred to a lower dilution giving negative results in another oyster. This is done in order to avoid the unnatural result that could follow from what is probably an unequal distribution of the bacteria in the shell water. This recession of numerical values, however, should not be carried beyond the point where the number of such recessions is greater than the number of instances where other oysters in the series failed to give positive B. coli results.

As an example of the method of obtaining the score for B. coli, the following illustration is given.

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[1] Where the term B. coli is used it refers in all cases to bacteria of the B. coli group and not to the specific prototype.

RESULTS OF B. COLI TESTS IN DILUTIONS INDICATED.

| Oysters. | 1 c.c. | 0.1 c.c. | 0.01 c.c. | Numerical value. |
|---|---|---|---|---|
| 1 | + | + | 0 | 10 |
| 2 | + | + | 0 | 10 |
| 3 | + | + | 0 | 10 |
| 4 | + | 0 | 0 | 10 (not 1) |
| 5 | + | + | + | 10 (not 100) |
| | | | | Score, 50 |

**Examination of Oysters Removed from the Shell, or Shucked Stock.**—The procedure specified for oysters in the shell shall be followed, but attention is called to the fact that higher dilutions than 100 c.c. are usually required. Triplicate fermentation tubes shall be inoculated from end dilutions of the sample.

*Statement of Results.*—The results of the bacteriological examination of the opened oysters or shucked stock shall be expressed in the same way as that specified for oysters in the shell, except that in the calculations of B. coli, rating the values for the results of the fermentation tests after confirmation shall be recorded for each of the inoculations of each dilution. In order that the rating from these triplicate tests may be compared with that obtained from testing five oysters in the shell, the sum of the values for the triplicate tests shall be multiplied by $\frac{5}{3}$. If, instead, the sum is divided by 3, the result will give approximately the number of B. coli per cubic centimeter.

**Clams and Other Shellfish.**—The methods of examining clams and shellfish other than oysters shall be those given above. Certain modifications are necessary in the method of handling the sample and the opening of the shells, etc.

Clams are more likely to lose water during transportation than oysters. It is therefore necessary to take greater precautions to separate different samples of clams from each other than in the case of oysters.

# CHAPTER LI.

# THE SOIL BACTERIA AND THEIR FUNCTIONS. SEWAGE BACTERIA. BACTERIA IN INDUSTRIES.

## SOIL BACTERIA.

THE bacteria in the soil belong to many varieties. Some varieties are only accidentally present, being due to the contamination of the earth with the bacteria contained in animal feces and other waste products. The majority, however, pass their life and reproduce themselves chiefly or wholly in the soil. Many of these varieties have most important functions to perform in continuing the earth's food supply. Without them plant food, and therefore animal food, would cease to exist. Some make available for plants the carbon, nitrogen, hydrogen, and other compounds locked up in the dead bodies of animals and plants. Others construct food for plants from the gases of the air and the inorganic elements of the earth which in their simpler forms were not available.

The bacteria, together with the other somewhat less important microscopic plants and animals, thus form a vital link in the earth's life cycle of plants and animals. The bacteria in the soil require for their activities food, moisture, and a proper temperature. Some soil bacteria grow at or below a freezing temperature. They may be present to the extent of many millions in a single gram of rich loam, while in an equal quantity of sand they may be almost absent.

The various species associated together in the soil flora influence each other. Thus anaërobic bacteria are enabled to grow because of associated aerobes using up the free oxygen, while other species make assimilable substances not usable by others.

**The Splitting up of Carbon Compounds.**—The plants form starch, and from it cellulose, woods, fats, and sugar. These substances once formed cannot be utilized by other generations of plants. Some of these are transformed in the bodies of animals, but the largest percentage await the activities of the microörganisms. The sugars and starches usually undergo an alcoholic fermentation excited by the yeasts and moulds with the production of alcohol and carbon dioxide or an acid fermentation excited by bacteria with the production of acids and frequently of carbon dioxide.

Cellulose which is so resistant to decay is attacked by certain varieties of bacteria which are abundant in the soil. They act both in the presence and absence of free oxygen. Moulds also act on cellulose. Carbon dioxide, marsh gas, and other products are produced. Wood is apparently first attacked by the fungi and only later by other microörganisms.

These bacteria are carried into the intestines and act upon cellulose and other substances.

**The Decomposition of Nitrogenous Compounds.**—Plants obtain their nitrogen chiefly in the form of nitrates. The small amount of usable nitrogen in the soil must be constantly replenished. This must either come from the nitrogen forming a part of protein materials or from the free nitrogen in the air.

The animals utilize the plant proteins and reduce them to much simpler compounds, such as urea, but even these are not suitable for plant use. We now know that microörganisms are employed to break compounds into simpler compounds and also to utilize the nitrogen of the air.

**Decomposition.**—This process is to some extent carried out through the agency of moulds and other fungi but it is chiefly due to the activities of bacteria. When this process is carried on in the absence of oxygen it is incomplete, giving rise to substances with unpleasant odors, such as $H_2S$, $NH_3$ and $CH_4$. This is called putrefaction. When oxygen is freely accessible more complete decomposition occurs with such end-products as $CO_2$, N and $H_2O$. These two processes, putrefaction and complete decay, cannot be sharply separated, as the second usually follows the first. The varieties of organisms causing these changes are many. Some groups will be found chiefly in decaying vegetable substances, others in animal tissues. They include all morphological forms of bacteria as well as yeasts and higher fungi. These forms exist everywhere in nature, although in various degrees, so that every bit of dead organic matter is sure to be decomposed if only moisture and warmth are present. The *B. subtilis* and *B. proteus vulgaris* groups are well-known laboratory bacteria that are commonly found among decomposing materials. *B. proteus* is described under Pathogenic Bacteria.

**B. subtilis, or hay bacillus.**

**The type form** has the following characteristics (Fig. 212):

**Source and Habitat.**—Hay, straw, soil, dust, milk, etc.

**Morphology.**—Short, thick rods with round ends, sometimes form threads; sometimes also chains of long rods, short rods and coccus forms. $0.8\mu$ to $1.2\mu$ broad, $1.3\mu$ to $3\mu$ long. Often united in strings and threads.

**Staining Reaction.**—Stains by Gram's method.

**Capsule, Flagella, Motility.**—Bacilli possess a thin capsule and many flagella which are long and numerous; short forms actively motile; threads immobile.

**Spore Formation.**—Oval spores formed in presence of air germinating at right angles to long diameter. Spores are set free in about twenty-four hours, size $1.2\mu$ by $0.6\mu$; widely distributed in nature, dust, air, excreta, etc. (Plate III, Fig. 23).

**Biology: Cultural Characters (Including Biochemical Features).—Bouillon.**—Uniformly cloudy growth with marked pellicle, wrinkled and thick; copious spore formation.

**Gelatin Plates and Tubes.**—Saucer-like depressions; colonies have granular centers and folded margins. Surface growth in stab cultures is whitish-gray; colonies sink on liquefaction of medium; liquefaction progresses in a cylindrical form, and a thick white scum is formed.

**Agar Plates and Tubes.**—Small, irregular, grayish-white colonies; moist glistening growth along needle track in stab cultures.

The bacteria in taking certain atoms from the molecules utilized in their growth leave the other atoms to enter into new relations and form new compounds. The actual products will depend on the decaying substance, the variety of bacteria and the conditions present.

**Nitrification.**—This is a process of oxidation by which through bacterial activities ammonia compounds are changed to nitrates and thus rendered utilizable by plants. This change is accomplished in two stages: first, the ammonia is oxidized to nitrite and, second, to nitrate. The nitrates are taken up by the plant roots from the soil. The bacterial nature of these changes were discovered in 1877 by two French investigators, Schlosing and Muntz. They noted that fermenting sewage after a time lost its ammonia and gained in nitrates, but that if the sewage was treated with antiseptics, so that fermentation ceased, no such change occurred. Warrington first and Winogradsky later more thoroughly investigated the bacterial cause of these changes. The latter by means of silica jelly, which contained no organic matter, was able to isolate two varieties of cocci, one in Europe and the other in America, which were able to change ammonia to nitrites. He called the one *nitrosomonas* and the other *nitrosococcus*. They are capable of acting on almost any ammonia salt. One variety of organisms capable of changing nitrites to nitrates was isolated, and this bacillus he called *nitrobacter*. These are small, slightly elongated bacilli. These bacteria are remarkable in that in pure cultures very small amounts of organic matter in the media act as antiseptics. They appear to be able to depend on mineral substances for their food. These bacteria are extremely important, for the plants take up most of their nitrogen in the form of nitrates. These changes are mostly produced in the surface soil. If the reaction of the soil becomes acid growth ceases. Soil bacteriologists are studying the nitrifying power of different types of soil under identical conditions. The processs being one of oxidation the access of air is necessary.

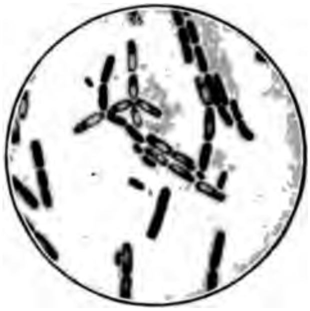

Fig. 212.—Bacillus subtilis with spores. Agar culture. Stained with gentian violet. × 1000 diameters. (Fränke.)

**Denitrification.**—This is a reducing process. The nitrate is made to yield up a part or all of its oxygen and thus becomes changed to nitrites and to ammonia and even to free nitrogen. The partial change does not rob the soil of its available nitrogen as does the total change, for the nitrites and ammonia may be changed by the nitrifying bacteria to nitrates. These bacteria exist normally in most soils and are especially abundant in manure. There are three different types of nitrogen reduction: (1) The reduction of nitrates to nitrites and ammonia. (2) The reduction of nitrates and nitrites to gaseous oxides of nitrogen. (3) The reduction of nitrites with the development of free nitrogen gas.

**Nitrogen-fixing Bacteria.**—Hellbrigel, in 1886, demonstrated that certain plaints were able to use the nitrogen of the air and this apparently through the aid of bacteria growing in their roots. These root bacteria are named *B. radicicola.* They produce enlargement (tubercles) on the roots.

According to Ball,[1] there is no reasonable doubt but that *B. radicicola* can and usually does remain active for very long periods in soil devoid of leguminous vegetation. Furthermore, the bacterium diffuses at a very considerable rate through soils that are in proper condition; therefore if a soil should be found lacking the organism, it is illogical to attempt to introduce it artificially without having first made the soil fit for the development of the bacteria.

It has not been shown by anyone that increased powers of resistance to unfavorable conditions of certain varieties are at all correlated with their enhanced "greed for nitrogen." Moreover, it is far from being proved that any one race or "physiological species" is really more virile than another. Greig-Smith[2] has shown that as many as three races are sometimes present in one and the same tubercle. Possibly, therefore, fixation of nitrogen may occur most rapidly only when two or more of these races are growing together.

Buchanan[3] has recently made a minute morphological study of *B. radicicola.* Some of his conclusions are as follows:

1. Considerable variation in the morphology of *B. radicicola* may be induced in artificial media by the use of appropriate nutrients. Of the salts of the organic acids, sodium succinate brings about the most luxuriant development and the production of the greatest variety of bacteroids.

2. *B. radicicola* in the roots of the legumes shows the same type of bacteroids as may be found in suitable culture media. On the other hand, there is little or no correspondence between the type of bacteroid produced in culture media by a certain organism and that produced in the nodule by the same form.

3. It is probable that the term *B. radicicola* includes an entire group of closely related varieties or species, which differ from each other to some degree in morphological characters.

4. The nodule organism resembles morphologically both the yeast and the bacteria. The difference between this form and those ordinarily included under the terms Bacillus and Pseudomonas justify the use of a separate generic name, *Rhizobium.*

In 1893 Winogradsky furnished proof that there are in the soil, bacteria which are outside of the plant roots performing the same function as those within the roots. These bacilli he called *Clostridium pasteurianum.* They are anaërobic and produce spores. Their power to fix nitrogen is increased in presence of sugar and lessened in presence of nitrogenous substances.

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[1] Centralbl. f. Bakt., etc., 1909, Abt. II, xxiii, 47.

[2] Jour. Soc. Chem. Indust., 1907, No. 7.

[3] Centralbl. f. Bakteriol., etc., 1909, Abt. II, xxiii, 59.

Beijerinck, in 1901, described two aërobic species of nitrogen-fixing bacteria. Later Bailey,[1] described three additional species. These were called *Azotobacter*. These studies have already led to the inoculation of soils and to the investigation of the kind of soils and crops best fitted for the growth of these bacteria. Many impoverished soils have already been greatly improved. There are probably many other varieties of bacteria capable of fixing nitrogen, because one can hardly examine the roots of any leguminous plants without finding different kinds of tubercles. The use of seed inoculated with the special variety of bacteria suitable for the plant and the soil is already largely practised.

**Bacteria and Soil Minerals.**—Some of the bacterial products act upon the inorganic constituents of the soil. The carbon dioxide and the organic acids act upon compounds of lime and magnesia, practically insoluble in water, to form more soluble substances. The same is true of the rock phosphates, the silicates, of potassium sulphates, etc.

Scientific farming is beginning to make use of the knowledge already acquired, and there is reason to hope that great practical advantages will flow from the investigation of the relation of bacteria to soil exhaustion and replenishment.

The effect of excessive bacterial development appears at times to be harmful to the soil. Each crop seems to favor the growth of certain varieties, and the exhaustion of the soil which follows the constant raising of the same crop is now suspected to be due in part at least to the continuance of a few restricted species of bacteria in the soil, which failing to produce all the necessary substances for the nutrition of the special crop, vegetation suffers, or again the bacteria finally entirely dissipate substances already in the soil necessary to growth.

The application of manure not only adds food for plant life, but also countless numbers of bacteria which make the food more available. The greatest number of bacteria are contained a little below the surface of the soil, where they are protected from drying and sunlight and are in contact with oxygen and with the roots and other food of the superficial soil.

## BACTERIA IN SEWAGE.

The materials which flow from our sewers are a menace to public health, mainly because they so frequently contain pathogenic bacteria. The other products of men and animals are offensive, but rarely concentrated enough in drinking water to be appreciably deleterious. Sewage can be made harmless by being sterilized, but can be freed from offense only by the destruction of organic matter. This, except when chemical precipitants are used, is almost wholly obtained through bacterial processes. The purifying value of soil has long been recognized. This is largely due to the action of the soil bacteria.

In 1895 the Englishman, Cameron, introduced the "septic tank," which was a covered cemented pit. The sewage admitted at the bottom flowed out at the top, after about twenty-four hours' subjection to anaërobic conditions. The anaërobic bacteria during this time f the organic matter energetically, liquefy it, and develop abunda The knowledge that soil and sand filters act not only mecha but also and perhaps chiefly bacteriologically, having been ac intermittent soil filtration was established as one of the best m bacteriologically purifying sewage. The sewage is conducted beds, allowed to pass through, and then after a few hours again on. The purification is based chiefly on the action of the aërobic b in the upper layers of the soil or sand. The best practical res obtained by combining the two processes: first the anaërobic tre is used to break down the solid materials, and then the inter sand filtration to oxidize the compounds and render these p harmless. With low temperatures the chemical changes are ver lessened and the filter beds act more like pure mechanical filters.

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[1] Bacteria in Relation to Country Life.

The anaërobic bacteria change the protein substances into chemical compounds, among which is ammonia. The carboh are changed into gaseous compounds, acids, etc. The gases are nitrogen, carbon dioxide, and marsh gas. The bacterial chang duced in sewage poured on contact beds made of coarse coke, cl or other material act much as in the sand filters after the filtr

**Varieties of Bacteria in Filter Beds and Septic Tanks.**—The tanks all contain spore-bearing bacilli which destroy cellulose, that attack nitrogenous compounds. The cocci are in a mi The filter beds have a number of small, non-sporebearing bacilli of these change ammonia into nitrites and nitrates. There a denitrifying bacteria. As before mentioned, the bacterial effi of the bed is increased with suitable temperature and much le with low temperature.

**Sewage Farming.**—The action of bacteria is utilized in the br up of sewage which has been distributed over fields. The amo sewage which can be poured on a certain area is limited. On of land can usually take care of the sewage from one hundred p If too much is poured on, it runs off unpurified or clogs the soi prevents the access of oxygen to aërobic bacteria. In warm w evaporation and bacterial activities are much greater than i weather. So far as experience shows, those who eat vegetable these small farms contract no disease from them.

## THE PRESERVATION OF FOODS AGAINST DECOMPOSITI BY MICROÖRGANISMS.

The preservation of foods against decomposition by bacteria, moulds, and higher fungi is obtained by using processes whi prevent the growth of microörganisms. Drying, exposure to smoke with consequent absorption of creosote, the addition and sugar, of acids such as vinegar, spices, germicides such as l acid, formaldehyde, all are familiar methods of making foods uns for bacterial growth.

Instead of using food preserved by drying or chemicals, products may be kept at temperatures too low for bacterial growth. Cold storage of meats, eggs, vegetables, etc., is now common.

The sterilization of food substances by heat with protection from infection afterward is made use of extensively in the canning of meats, fruits and vegetables. Care must be taken that absolutely all bacteria are killed, for otherwise decomposition will finally occur. The products of decomposition are now known to be much less deleterious than they were formerly thought to be.

**Bacterial Fermentation in Relation to Miscellaneous Products.**—Pasteur, in 1857, explained the process of fermentation as due to the action of microörganisms. He demonstrated that the change of sugar into lactic acid only occurred when living bacilli were present. If the fluid was sterilized the fermentation ceased. He stated that "organic liquids do not alter until a living germ is introduced into them." When the action is direct we speak of an organized ferment; when it is indirect, that is, due to the cell product, we call it an unorganized soluble ferment or enzyme. Similar enzymes are produced by the cells of the animal tissues, such as ptyalin, pepsin, and trypsin. Pasteur's work led to the conclusion that the different fermentations were due to different varieties of organisms. The major part of fermentation is due to yeast. Some important fermentations are due to bacteria and a few to the moulds.

**Wines and Beers.—Alcoholic Fermentation.**—If there is a development of the yeast cells in a solution of grape-sugar we have a fermentation of the sugar with a final development of alcohol and carbon dioxide. It is thus that beers and wines are developed. When the carbohydrate is in the form of starch this is first converted into sugar and then later into the final products. If the sugar is in the form of saccharose, it is first changed by the yeast ferments to glucose. In all these three forms of fermentation the sugar is changed into alcohol and carbonic acid. When the alcohol reaches about 13 per cent. it stops further fermentation. These yeasts called saccharomyces comprise a number of distinct varieties, some of which are cultivated while others, called "wild yeasts," propagate themselves. The distillery, brewery, and wine industries each make use of special yeasts and special conditions. The rising of bread is one of the most common uses of fermentation by yeast. The yeast acts upon the sugar made by the diastase from the starch. The resulting $CO_2$ and alcohol create myriads of little bubbles in the dough.

*Diseases of Beer and Wines.*—Hansen, Pasteur, and others demonstrated that the spoiling of beers and wines was due to the development of varieties of bacteria and yeasts which produce different kinds of fermentation from that desired. These produce alterations in flavor, bitterness, acidity.

**Vinegar Making.**—Vinegar is made from some weak alcoholic solution by the union of alcohol with oxygen. This oxidation can be brought about by a purely chemical process. When vinegar is formed in the usual way bacteria are essential. The scum on the surface of the fermenting alcohol is a mass of microörganisms. The mother of vinegar was named mycoderma by Pearson. Kutzing showed that this was composed of living cells. Hansen proved these to be bacteria. We now know there are many varieties of bacilli capable of producing this fermentation. Each variety has its own optimum temperature and differs in the amount of acid it produces. Most of these have the peculiarity of growing at high temperatures into long threads without any traces of division. At low temperatures they produce long threads with swollen centers. The usual vinegar is made by using the variety of bacilli prevalent in the surroundings, but the custom is growing of adding to the pasteurized alcoholic solution the special variety desired in pure culture.

**Sauerkraut.**—This is cabbage leaves shredded, slightly fermented, and prevented from decay by the lactic acid bacteria. At first both yeasts and bacteria increase together, but with the increase in acidity all growth ceases. Putrefaction is prevented by the same cause. The lactic acid bacteria are the same as those found in sour milk.

**Ensilage.**—The fermentation is believed to be due partly to enzymes in the corn tissues and partly to bacterial action. The first changes are due chiefly to the enzymes.

**The Curing of Tobacco.**—The curing of tobacco is apparently due partly to bacterial processes and partly to the action of leaf enzymes.

**The Bacterial Diseases of Plants.**—These are probably as serious and varied for plants as for animals. The pear blight, the wilt disease of melons, the brown rot of tomatoes the black rot of cabbages are examples. These plant diseases can be communicated by means of the pathogenic pure cultures of bacteria experimentally just as readily as animal diseases by their specific bacteria.

# CHAPTER LII.

## THE DESTRUCTION OF BACTERIA BY CHEMICALS. PRACTICAL USE OF DISINFECTANTS.

MANY substances, when brought in contact with bacteria, combine with their cell substance and destroy the life of the bacteria. While in the vegetative stage bacteria are much more easily killed than when in the spore form, and their life processes are inhibited by substances less deleterious than those required to destroy them.

Bacteria, both in the vegetative and in the spore form, differ among themselves considerably in their resistance to the poisonous effects of chemicals. The reason for this is not wholly clear, but it is connected with the structure and chemical nature of their cell substance.

Chemicals in sufficient amount to destroy life are more poisonous at temperatures suitable for the best growth of bacteria than at lower temperatures, and act more quickly upon bacteria when they are suspended in fluids singly than when in clumps, and in pure water rather than in solutions containing organic matter. The increased energy of disinfectants at higher temperatures indicates in itself that a true chemical reaction takes place. In estimating the extent of the destructive or inhibitive action of chemicals the following degrees are usually distinguished:

1. The growth is not permanently interfered with, but the pathogenic and zymogenic functions of the organism are diminished—*attenuation*. This loss of function is usually quickly recovered.

2. The organisms are not able to multiply, but they are not destroyed—*antiseptic* action. When transferred to a suitable culture fluid free of the disinfectant these bacteria are capable of reproduction.

3. The vegetative development of the organisms is destroyed, but not the spores—incomplete or complete sterilization or disinfection, according as to whether spores are present in the organisms exposed and as to whether these spores are capable of causing infection.

4. Vegetative and spore forms are destroyed. This is complete *sterilization* or *disinfection*.[1]

The methods employed for the determination of the germicidal action of chemical agents on bacteria are, briefly, as follows:

If it is desired to determine the minimum concentration of the chemical substance required to produce complete inhibition of growth we proceed thus: A 10 per cent. solution of the disinfectant is prepared and 1 c.c.,

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[1] Disinfection strictly defined is the destruction of all organisms and their products which are capable of producing disease. Sterilization is the destruction of all saprophytic as well as parasitic bacteria. It is not necessary in most cases to require disinfectants to be capable of sterilizing infected materials containing spores, for there are but few varieties of pathogenic bacteria which produce spores.

0.5 c.c., 0.3 c.c., etc., of this is added to 10 c.c. of
or bouillon, or, more accurately 10 c.c. minus t
added, in so many tubes. The tubes then conta
cent., 0.3 per cent., and 0.1 per cent. of the d
medium in the tubes is then inoculated with a p
test bacterium. The melted agar and gelatin r
and allowed to remain in the tubes, and watched
takes place, or the contents of the tubes may
dishes, where the development or lack of develo
the number can be observed. If no growth occurs
lower dilutions are tested. Bacteria that have b
in any way will be inhibited by much weaker
than will vigorous cells. The same test can b
containing only spores.

If it is desired to determine the degree of con
the destruction of vegetative development, the
is cultivated in bouillon, and into each of a seri
definite amount of diluted culture from which
have been filtered; to these a definite amount
different percentages of the disinfectant is added
five, ten, fifteen, and thirty minutes, one hour, and
loopful of the mixture is taken from each tube
c.c. of fluid agar or gelatin, from which plate cult
ever it is probable that the antiseptic power c
proaches somewhat the germicidal, it is necessar
series of tubes from the first so as to decrease still
antiseptic carried over. The results obtained are
per cent. of the disinfectant in watery solution anc
the organism in twenty minutes, z per cent. at the
in one minute, and so on. If there be any doubt w
disinfectant carried over with the platinum loops
gelatin unsuitable for growth, thus falsifying resu
extreme accuracy is desired, should be made by
have been somewhat enfeebled by slight contact v
fluid to which a similar trace of the disinfectant h
strength of the disinfectant is to be tested for diffe
be tested in these substances or their equivalent,

The disinfectant to be examined should alw
inert fluid, such as water; if on account of its bei
is necessary to use another solvent, control experi
to determine its action on the organism. Someti
corrosive sublimate, the chemical unites with the
an unstable compound, which inhibits the growth
time before destroying it. If this compound is
media it will probably not be in the body. In son
to break up this union and note then whether th
dead. With corrosive sublimate the bacteria prol
minutes after the union occurs.

In the above determinations the absolute strength of the disinfectant required is considerably less when culture media poor in albumin are employed than when the opposite is the case Cholera spirilla grown in bouillon containing no peptone or only 0.5 per cent. of peptone are destroyed in half an hour by 0.1 per cent. of hydrochloric acid; grown in 2 per cent. peptone bouillon, their vitality is destroyed in the same time on the addition of 0.4 per cent. HCl. In any case the organisms to be tested should all be treated in exactly the same way and the results accompanied by a statement of the conditions under which the tests were made. It is becoming the custom to state the power of a disinfectant in terms of comparison with pure carbolic acid. A substance which had the same destructive power in a 1 to 1000 solution as carbolic acid in a 1 to 100 solution would be rated as of a strength ten times that of carbolic acid.

The following table gives the results and methods used in an actual experiment to test the effect of blood serum upon the disinfecting action of bichloride of mercury and carbolic acid upon bacteria.

TEST FOR THE DIFFERENCE OF EFFECT OF BICHLORIDE OF MERCURY AND CARBOLIC ACID SOLUTION ON TYPHOID BACILLI IN SERUM AND IN BOUILLON.

| Time. | 1′ | 3′ | 5′ | 10′ | 20′ | 30′ | 45′ | 1 hr. | 1½ hrs. | 2 hrs. | Strength of solution. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A. serum . . 2.5 c.c.<br>$HgCl_2$ sol. 1:1000 2.5 c.c.<br>Typhoid broth culture | + | + | + | – | – | – | – | – | – | – | Equals 1:2000 bichloride. |
| B. bouillon . 2.5 c.c.<br>$HgCl_2$ sol. 1:1000 2.5 c.c.<br>Typhoid broth culture | – | – | – | – | – | – | – | – | – | – | Same. |
| C. serum . . 2.5 c.c.<br>Carbolic sol. 5% 2.5 c.c.<br>Typhoid broth culture | + | + | – | – | – | – | – | – | – | – | Equals 2½% carbolic acid. |
| D. bouillon . 2.5 c.c.<br>Carbolic sol. 5% 2.5 c.c.<br>Typhoid broth culture | + | – | – | – | – | – | – | – | – | – | Same. |

– Indicates total destruction of bacteria with no growth in media.
+ Indicates lack of destruction of bacteria with growth in media.

## THE STANDARDIZATION OF DISINFECTANTS.

Rideal and Walker were the first to urge a useful method for standardizing disinfectants.

In carrying out the test the various factors must be carefully controlled, thus: *Time:* this should be constant, the strength of the disinfectant being the variant. *Test organisms:* a standard culture of the typhoid bacillus (Hopkins' strain) is used to avoid any variations due to the different degrees of resistance of various strains. The culture should be subinoculated three days before used. *Medium:* a standard meat-extract broth 1.5 per cent. acid to phenolphthalein; 10 c.c. to a tube is employed. *Temperature:* this test is done at 20° C. This is important, as the germicidal activity increases with the temperature. *Constant amount of culture used:* 0.1 c.c. of the twenty-four-hour broth culture is added to 5 c.c. of the disinfectant solution. This is more accurate than the drop method. *Amount inoculated:* it is essential that the same amount be inoculated from each dilution. Platinum loops made of 23 United States gauge wire, the loops being 4 mm. in diameter are employed. Several are used, being left on a rack after sterilization so as to be cold when needed. The loop is bent at an angle of 45° to the shank.

The actual test is carried out as follows: A 5 per cent. carbolic solution (phenol C. P), is prepared and standardized by bromine titration. From this freshly prepared 1 to 90 to 1 to 100 and 1 to 110 dilutions are made as needed. The necessary dilutions of germicide are then prepared. Wide jumps in the dilutions are made in the first test and then narrowed as the limits of the disinfectant are determined.

Five test-tubes are arranged in a row in a water-bath at 20° C., and the solutions added in 5 c.c. amounts. Time must be allowed for the solution to reach 20° C. If the bath be large enough, little attention is needed to keep the temperature constant. The culture having been brought to 20° C, is then added in 0.1 c.c. amounts and the tubes shaken, an interval of thirty seconds allowed between each tube. Subinoculation of the first tube is then made after thirty seconds, which gives an interval of two and a half minutes after inoculation. The tubes are then subinoculated in order at thirty-second intervals, giving an interval for each of two and one-half minutes after inoculation, and starting at the first, gives an interval of five minutes, etc.

It is possible to use ten tubes, as each step can be done in less than fifteen seconds if properly arranged, allowing a much wider range for each test. It is not necessary to keep the cotton plugs in the tube during the operation nor to remove the tubes from the bath to obtain the loopful for inoculation. The loop is plunged to the bottom, care being taken not to touch the sides of the tubes, and care should also be taken that a loopful is carried away each time. The broth tubes are incubated for forty-eight hours and then examined for growth.

The following are given as two examples:

| | | Time of exposure: | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Sample. | Dilution. | 2.5 min. | 5 min. | 7.5 min. | 10 min. | 12.5 min. | 15 min. | Phenol coefficient. |
| Phenol . . . | 1 to 90 | + | – | – | – | – | – | 100)550 |
| | 1 to 100 | + | + | + | – | – | – | 5.5 |
| Disinfectant A . | 1 to 450 | + | + | – | – | – | – | |
| | 1 to 550 | + | + | + | – | – | – | |
| | 1 to 600 | + | + | + | + | – | – | |
| Phenol . . . | 1 to 100 | + | + | + | – | – | – | 110)650 |
| | 1 to 110 | + | + | + | + | + | – | 5.191 |
| Disinfectant A . | 1 to 600 | + | + | + | + | – | – | |
| | 1 to 650 | + | + | + | + | + | – | |
| | 1 to 700 | + | + | + | + | + | + | |

These tables not only serve as an example, but also show that unless many repetitions of the tests are made and averaged, considerable variations in the results may be obtained. A report of 5.5 or 5.1 is equally accurate in the test here reported of the Rideal-Walker method, even with certain improvements added. With practice, and by selection of the dilutions to be employed, the operator evidently can regulate the time factor so that fairly uniform results are obtainable. On the other hand, it not infrequently happens that if more than one carbolic dilution is employed, more than one time period is open to comparison. For these reasons Anderson and McClintic have modified the test by setting two time limits two and one-half and fifteen minutes and taking the average. The following is an example:

| Sample. | Dilution. | Time of exposure. | | | | | | Phenol coefficient. |
|---|---|---|---|---|---|---|---|---|
| | | 2.5 min. | 5 min. | 7.5 min. | 10 min. | 12.5 min. | 15 min. | |
| Phenol . . . | 1 to 80 | − | − | − | − | − | − | $\frac{375}{80}$ $\frac{650}{110}$ |
| | 1 to 90 | + | − | − | − | − | − | |
| | 1 to 100 | + | + | + | − | − | − | |
| | 1 to 110 | + | + | + | + | + | − | |
| Disinfectant A . | 1 to 350 | − | − | − | | | | |
| | 1 to 375 | − | − | − | | | | |
| | 1 to 400 | + | − | − | − | | | $\frac{4.69 + 5.91}{2} = 5.30$ |
| | 1 to 425 | + | + | − | − | − | − | |
| | 1 to 450 | + | + | − | − | − | − | |
| | 1 to 500 | + | + | − | − | − | − | |
| | 1 to 550 | + | + | + | − | − | − | |
| | 1 to 600 | + | + | + | + | − | − | |
| | 1 to 650 | + | + | + | + | + | − | |
| | 1 to 700 | + | + | + | + | + | + | |
| | 1 to 750 | + | + | + | + | + | + | |

Disinfectants vary widely in their germicidal properties, depending on whether organic matter is present or not. As under practical conditions organic matter is usually present, it is of some importance to know how far organic matter decreases the efficiency.

For the purpose of obtaining comparable results, Anderson and McClintic have suggested the use of peptone 10 per cent. and gelatin 5 per cent. in distilled water. One part of the culture is mixed with 10 parts of the organic solution, 1.1 c.c. being then added to a series of dilution tubes containing 4 c.c. In determining the coefficient allowance must be made for the added amount of organic matter.

The modified methods of Anderson and McClintic[1] are called the "hygienic laboratory phenol coefficient," with or without organic matter. Any organic matter may be used in the test to approach the special conditions under which a disinfectant is to be used.

In comparing the value of disinfectants the cost as well as the coefficient must be considered. This is best stated in terms of the relative cost of 100 units of efficiency as compared with pure phenol = 100, thus:

$$\frac{\text{Cost of disinfectant per gallon}}{\text{Cost of phenol per gallon}} \; (= \text{Cost ratio}) \div \frac{\text{Coefficient of disinfectant}}{\text{Coefficient of phenol (1).}}$$

$$(= \text{the efficiency}), \times 100 = \text{relative cost per 100 units.}$$

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[1] See Hygienic Laboratory Bulletin No. 82, for further details and apparatus for simplifying the steps of the test.

**Antiseptic Value.**—With certain disinfectants there is sufficient of the disinfectant carried over by the loop to exert antiseptic action and growth does not occur. If this is not taken into consideration a disinfectant will be given an excessively high coefficient. No satisfactory method has been devised to avoid this difficulty. The inoculated broth tube may be shaken and a loop or more inoculated from it to second broth tube, in this way diluting the disinfectant still further.

Chick[1] has attempted to overcome the difficulty in the case of mercury-containing disinfectants by adding 0.2 c.c. of a saturated watery solution of hydrogen sulphide to each tube of broth.

Many substances which are strong disinfectants become altered under the conditions in which they are used, so that they lose a portion or all of their germicidal properties; thus, quicklime and milk of lime act by means of their alkali and are disinfecting agents only so long as sufficient calcium hydroxide is present. If this is changed by the carbon dioxide of the air into carbonate of lime it becomes harmless. Bichloride of mercury and many other chemicals, form compounds with many organic and inorganic substances, which, though still germicidal, are much less so than the original substances. Solutions of chlorine, peroxides, etc., when in contact with an excess of organic matter soon become inert because of the chemical compounds formed.

**The Disinfecting Properties of Inorganic Compounds.**—**Bichloride of Mercury.**—This substance, which dissolves in 16 parts of cold water, when present in 1 part in 100,000 in nutrient gelatin or bouillon, inhibits the development of most forms of bacteria. In water 1 part in 50,000 will kill many varieties in a few minutes, but in bouillon twenty-four hours may be needed. With organic substances its power is lessened, so that 1 part to 1000 may be required. Most spores are killed in 1 to 500 watery solution within one hour. Corrosive sublimate is less effective as a germicide in alkaline fluids containing much albuminous substance than in watery solution. In such fluids, besides loss in other ways, precipitates of aluminate of mercury are formed which are at first insoluble, so that a part of the mercuric salt does not really exert any action. In alkaline solutions, such as blood, blood serum, pus, sputum, tissue fluids, etc., the soluble compounds of mercury are converted into oxides or hydroxides.

For ordinary use, where corrosive sublimate is employed, solutions of 1 to 500 and 1 to 2000 will suffice, when brought in contact with bacteria, to kill the vegetative forms within from one to twenty minutes, the stronger solution to be used when much organic matter is present.

Mercuric chloride volatilizes slowly and it is better to wash off walls after use of bichloride solutions. Solutions of this salt should not be kept in metal receptacles. Mercuric chloride solution has disadvantages in that it corrodes metals, irritates the skin, and forms almost inert compounds with albuminous matter. In order to avoid accidents, solutions of this odorless disinfectant should be colored by some dye.

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[1] Journal of Hygiene, 1908, viii, 654.

**Biniodide of Mercury.**—This salt is very similar in its effect to the bichloride.

**Nitrate of Silver.**—Nitrate of silver in watery solution has about one-fourth the value of the bichloride of mercury as a disinfectant, but nearly the same value in inhibiting growth. In albuminous solutions it is equal to bichloride of mercury. Compounds of silver nitrate and albuminous substances have been used because of the absence of irritative properties combined with moderate antiseptic power.

**Sulphate of Copper.**—This salt has about 50 per cent. of the value of mercuric chloride. It has a quite remarkable affinity for many species of algæ, so that when in water 1 to 1,000,000 it destroys many forms; 1 to 400,000 destroys typhoid bacilli in twenty-four hours when the water has no excessive amount of organic material. It is not known to be poisonous in this strength, so that it can be temporarily added to water supplies.

**Sulphate of Iron.**—This is a much less powerful disinfectant than sulphate of copper. A 5 per cent. solution requires several days to kill the typhoid bacilli. It can only be considered as a mild antiseptic and deodorant.

**Zinc Chloride.**—This is very soluble in water, but is a still weaker disinfectant than copper sulphate.

**Sodium Compounds.**—A 30 per cent. solution of NaOH kills anthrax spores in about ten minutes, and in 4 per cent. in about forty-five minutes. One per cent. kills vegetative forms in a few minutes. Sodium carbonate kills spores with difficulty even in concentrated solution, but at 85° C. it kills spores in from eight to ten minutes. It is used frequently to cover metallic instruments. A 5 per cent. solution kills in a short time the vegetative forms of bacteria. Even ordinary soapsuds have a slight bactericidal as well as a marked cleansing effect. The bicarbonate has almost no destructive effect on bacteria.

**Calcium Compounds.**—Calcium hydroxide, $Ca(OH)_2$, is a powerful disinfectant; the carbonate, on the other hand, is almost without effect. The former is prepared by adding one pint of water to two pounds of lime (quicklime, CaO). Exposed to the air the calcium hydrate slowly becomes the inert carbonate. A 1 per cent. watery solution of the hydroxide kills bacteria which are not in the spore form within a few hours. A 3 per cent. solution kills typhoid bacilli in one hour. A 20 per cent. solution added to equal parts of feces or other filth and mixed with them will completely sterilize them within two hours.

**Effects of Acids.**—An amount of acid per liter which is equivalent to 40 c.c. of normal hydrochloric acid is sufficient to prevent the growth of all varieties of bacteria and to kill many. Twice this amount destroys most bacteria within a short time. The variety of acid makes little difference. Bulk for bulk, the mineral acids are more germicidal than the vegetable acids, but that is because their molecular weight is so much less. A 1 to 500 solution of sulphuric acid kills typhoid bacilli within one hour. A similar solution of hydrochloric acid is about one-third weaker, and acetic acid somewhat weaker still. Citric, tartaric, malic, formic, and salicylic acids are similar to acetic acid. Boric acid destroys the less resistant bacteria in 2 per cent. solution and inhibits the others.

**Gaseous Disinfectants.**—The germicidal action of gases is much more active in the presence of moisture than in a dry condition.

**Sulphur Dioxide** ($SO_2$).—Numerous experiments have been made with this gas owing to the fact that it has been so extensively used for the disinfection of hospitals, ships, apartments, clothing, etc. This gas is a much more active germicide in a moist than in a dry condition; due, no doubt, to the formation of the more active disinfecting agent—sulphurous acid ($H_2SO_3$). In a pure state anhydrous sulphur dioxide does not destroy spores, and is not certain to destroy bacteria in the vegetative form. Sternberg has shown that the spores of the *Bacillus anthracis* and *Bacillus subtilis* are not killed by contact for some time with liquid $SO_2$ (liquefied by pressure). Koch found that various species of spore-bearing bacilli exposed for ninety-six hours in a disinfecting chamber to the action of $SO_2$, in the proportion of from 4 to 6 per cent. by volume, were not destroyed. In the absence of spores, however, the anthrax bacillus in a moist condition, attached to silk threads, was found by Sternberg to be destroyed in thirty minutes in an atmosphere containing 1 per cent. by volume.

As the result of a large number of experiments with $SO_2$ as a disinfectant it has been determined that an "exposure for eight hours to an atmosphere containing at least 4 volumes per cent. of this gas *in the presence of moisture*" will destroy most, if not all, of the pathogenic bacteria in the absence of spores. Four pounds of sulphur burned for each 1000 cubic feet will give an excess of gas.

**Peroxide of Hydrogen** ($H_2O_2$).—This is an energetic disinfectant, and in 2 per cent. solution (about 40 per cent. of the ordinary commercial article) will kill the spores of anthrax in from two to three hours. A 20 per cent. solution of a good commercial hydrogen peroxide solution will quickly destroy the pyogenic cocci and other spore-free bacteria. It combines with organic matter, becoming inert. It is prompt in its action and not poisonous, but apt to deteriorate if not properly kept.

**Chlorine.**—Chlorine is a powerful gaseous germicide, owing its activity to its affinity for hydrogen and the consequent release of nascent oxygen when it comes in contact with microörganisms in moist condition. It is therefore a much more active germicide in the presence of moisture than in a dry condition. Thus, Fischer and Proskauer found that dried anthrax exposed for an hour in an atmosphere containing 44.7 per cent. of dry chlorine were not destroyed; but if the spores were previously moistened and were exposed in a moist atmosphere for the same time, 4 per cent. was effective, and when the time was extended to three hours 1 per cent. destroyed their vitality. The anthrax bacillus, in the absence of spores, was killed by exposure in a moist atmosphere containing 1 part to 2500 for twenty-four hours.

In watery solutions 0.2 per cent. kills spores within five minutes and the vegetative forms almost immediately. One part in one million is sufficient to destroy typhoid bacilli, in a water containing little organic matter, in the course of a few hours. In water with much organic matter a much greater amount is required.

**Chlorinated Lime (Called "Chloride of Lime").**—Chlorinated lime is made by passing nascent chlorine gas over unslaked lime. It should not contain less than 10 per cent. of available chlorine, and can now be obtained containing 30 per cent. It should have a strong odor of chlorine. Its efficacy depends on the chlorine it contains in the form of hypochlorites. The calcium hypochlorite is readily broken up into hypochlorous acid. A solution in water of 0.5 to 1 per cent. of chlorinated lime will kill most bacteria in one to five minutes, and 1 part in 100,000 will destroy typhoid bacilli in twenty-four hours. A 5 per cent. solution usually destroys spores within one hour. Chlorinated lime not only bleaches but destroys fabrics.

**The Hypochlorites (Labarraque's Solution).**—Solutions of hypochlorites are practically the same as solutions of chlorinated lime and are much more expensive.

**Bromine and iodine** are of about the same value as chlorine for gaseous disinfectants, in the moist condition; but, like chlorine, they are not applicable for general use in house disinfection, owing to their poisonous and destructive properties; they have a use in sewers and similar places.

**Trichloride of iodine** in 0.5 per cent. solution destroys the vegetative forms of bacteria in five minutes.

**Organic Disinfectants.**—**Alcohol** in 10 per cent. solution inhibits the growth of bacteria; absolute alcohol kills bacteria in the vegetative form in from several to twenty-four hours. According to Epstein, 50 per cent. alcohol (in water) has more germicidal power than any other strength, the power gradually diminishing with both stronger and weaker solutions.

**Formaldehyde.**—Formaldehyde, or formic aldehyde, was isolated by von Hoffmann in 1867, who obtained it by passing the vapors of methyl alcohol mixed with air over finely divided platinum heated to redness. The methyl alcohol is oxidized and produces formaldehyde as follows:

$$CH_3OH + O = CH_2O + H_2O$$

Formaldehyde is a gaseous compound possessed of an extremely irritating odor. At a temperature of 68° F. the gas is polymerized—that is to say, a second body is formed, composed of a union of two molecules of $CH_2O$. This is known as a paraformaldehyde, and is a white, soapy body, soluble in boiling water and in alcohol. Formaldehyde is sold in commerce as a clear, watery liquid containing from 33 to 40 per cent. of the gas and 10 to 20 per cent. of methyl alcohol, its chief impurity. If the commercial solution—ordinarily known in the trade as "formalin"—is evaporated or concentrated above 40 per cent., paraformaldehyde results; and when this is dried *in vacuo* over sulphuric acid a third body—trioxymethylene—is produced, consisting of three molecules of $CH_2O$. This is a white powder, almost insoluble in water or alcohol, and giving off a strong odor of formaldehyde. The solid polymers of formaldehyde, when heated, are again reduced to an active gaseous condition; ignited, they finally take fire and burn with a blue flame, leaving but little ash. When burned they have no germicidal properties.

Formaldehyde has an active affinity for many organic substances, and forms with some of them definite chemical combinations. It combines readily with ammonia to produce a compound called hexamethylene tetramine, which possesses neither the odor nor the antiseptic properties of formaldehyde. This action is made use of in neutralizing the odor of formaldehyde when it is desired to dispel it rapidly after disinfection.

Formaldehyde also forms combinations with certain anilin colors — viz., fuchsin and safranin—the shades of which are thereby changed or intensified. These dyes are tests for aldehydes. These are the only colors, however, which are thus affected, and as they are seldom used in dyeing, owing to their liability to fade, this effect is of little practical significance. The most delicate fabrics of silk, wool, cotton, fur, etc., are unaffected in texture or color by formaldehyde. Iron and steel are attacked, after long exposure, by the gas in combination with watery vapor; but copper, brass, nickel, zinc, silver and gilt work were not at all acted upon.

Formaldehyde unites with nitrogenous products of decay—fermentation or decomposition—forming true chemical compounds, which are odorless and sterile. It is thus a true deodorizer in that it does not replace one odor by another more powerful, but forms new chemical compounds which are odorless. Formaldehyde has a peculiar action upon albumin, which it transforms into an insoluble and indecomposable substance. It renders gelatin insoluble in boiling water and most acids and alkalies. It is from the property of combining chemically with the albuminoids forming the protoplasm of bacteria that formaldehyde is supposed to derive its bactericidal powers. Formaldehyde is an excellent preservative of organic products. It has been used for the preservation of meat, milk, and other food products; but, according to Trillat and other investigators, formaldehyde renders these substances indigestible and unfit for food. It has been successfully employed as a preservative of pathological and histological specimens.

There are no exact experiments recorded of the physiological action of formaldehyde on the human subject when taken internally. A 1 per cent. solution has been taken in considerable quantity without serious results; and trioxymethylene has been given in doses up to 90 grains as an intestinal antiseptic. According to Aronson rabbits and guinea-pigs allowed to remain for twelve and twenty-four hours in rooms which were being disinfected with formaldehyde gas were found to be perfectly well when the rooms were opened. On autopsy the animals showed no injurious effects of the gas. Others have noticed that animals, such as dogs and cats, which have accidentally been confined for any length of time in rooms undergoing formaldehyde disinfection occasionally died from the effects of the gas. Many observers, however, have reported that insects, such as roaches, flies, and bed-bugs are not, as a rule, affected. The result of these observations would seem to indicate that although formaldehyde is comparatively non-toxic to the higher forms of animal life, nevertheless a certain degree of caution should be observed in the use of this agent. It is important to remember that formaldehyde in gaseous form is practically inert as an insecticide except in extremely great concentrations.

The researches of Pottevin and Trillat have shown that the germicidal power of the gas depends not only upon its concentration, but also upon the temperature and the condition of the objects to be sterilized. As with other gaseous disinfectants—viz., sulphur dioxide and chlorine—it has been found that the action is more rapid and complete at high temperatures—*i. e.*, at 35° to 45° C. (95° to 120° F.)—and when the test objects are moist, than at lower temperatures and when the objects are dry. Still, it has been repeatedly demonstrated by actual experiment in rooms that it is possible to disinfect the surface of apartments and articles contained in them, under the conditions of temperature and moisture ordinarily existing in rooms even in winter, by an exposure of a few hours to a saturated atmosphere of formaldehyde gas. The results of numerous experiments have shown that in the air 2.5 per cent. by volume of the aqueous solution, or 1 per cent. by volume of the gas, are sufficient to destroy fresh virulent cultures of the common non-sporebearing pathogenic bacteria in a few minutes.

Stahl has shown that bandages and iodoform gauze can be kept well sterilized by placing in the jars containing pieces of a preparation of paraformaldehyde in tablet form containing 50 per cent of formaldehyde. The same experimenter has also succeeded in making carpets and articles of clothing germ-free by spraying them with 0.5 to 2 per cent. solution of formaldehyde for fifteen to twenty minutes without the color of the fabrics being in any way affected. The investigations of Trillat, Aronson, Pottevin, and others have shown that a concentration of $\frac{1}{10000}$ of the aqueous solution (40 per cent.), equal to $\frac{1}{25000}$ of pure formaldehyde was safe and sufficiently powerful to retard bacterial growth.

A 2 per cent. watery solution of formalin destroys the vegetative forms of bacteria within five to thirty minutes. In our experiments formalin has upon the vegetative forms about one-half the strength of pure carbolic acid.

**Chloroform** ($CHCl_3$).—This substance, even in pure form, does not destroy spores, although it kills bacteria in vegetative forms, even in 1 per cent. solution. Chloroform is used practically as an antiseptic in antitoxic sera and in blood serum for culture purposes. The chloroform is expelled from the serum by heating it to 55° C.

**Iodoform** ($CHI_3$).—This substance has but very little destructive action upon bacteria; indeed, upon most varieties it has no appreciable effect whatever. When mixed with putrefying matter, wound discharges, etc., the iodoform is reduced to soluble iodine compounds, which act partly by destroying the bacteria and partly by uniting with poisons already produced.

**Carbolic Acid** ($C_6H_5OH$).—Pure phenol crystallizes in long, colorless crystals. In contact with air it deliquesces. It has a penetrating odor, a burning taste, and is a corrosive poison. It is soluble at ordinary temperatures in about 15 parts of water. Carbolic acid dissolves in water with some difficulty and should be therefore thoroughly mixed. It is not destructive to fabrics, colors, metals, or wood, and does not combine as actively with albuminous matters as bichloride of mercury. It is therefore more suitable for the disinfection of feces, etc. A solution having 1 part to 1000 inhibits the growth of bacteria; 1 part to 400 kills the less resistant bacteria, and 1 part to 100 kills the remainder. A 5 per cent. solution kills the less resistant spores within a few hours and the more resistant in from one day to four weeks. A slight increase in temperature aids the destructive action; thus, even at 37.5° spores are killed in three hours. A 3 per cent. solution kills streptococci, staphylococci, anthrax bacilli, etc., within one minute. Carbolic acid loses much of its value when in solution in alcohol or ether. An addition of 0.5 HCl aids its activity. Carbolic acid is so permanent and so comparatively little influenced by albumin that it is widely used in practical disinfection even in place of more powerful substances.

**Cresol.**—Cresol [$C_6H_4(CH_3)OH$] is the chief ingredient of the so-called "crude carbolic acid." This is almost insoluble in water, and therefore of restricted value. Many methods are used for bringing it into solution so as to make use of its powerful disinfecting properties. With equal parts of crude sulphuric acid it is a powerful disinfectant, but it is, of course, strongly corrosive. An alkaline emulsion of the cresols and other productscontained in "crude" carbolic acid with soap is called creolin. It is used in 1 to 5 per cent. emulsions. It is fully as powerful as pure carbolic acid. Lysol is similar to creolin, except that it has more of the cresols and less of the other products. It and creolin are of about the same value.

**Tricresol.**—Tricresol is a refined mixture of the three cresols (metacresol, paracresol, and orthocresol). It is soluble in water to the extent of 2.5 per cent. and its disinfecting power is about three times as great as that of carbolic acid.

**Creolin.**—Creolin contains 10 per cent. of cresols held in solution by soap.

**Lysol.**—Lysol contains about 50 per cent. of cresols. It mixes with water in all dilutions.

**Oil of Turpentine,** 1 to 200, prevents the growth of bacteria.

**Camphor** has very slight antiseptic action.

**Creosote** in 1 to 200 kills many bacteria in ten minutes; 1 to 100 failed to kill tubercle bacilli in twelve hours.

**Essential Oils.**—Cardéac and Meumir found that the essences of cinnamon, cloves, thyme, and others killed typhoid bacilli within one hour. Sandalwood required twelve hours.

## AVERAGE ACTION OF REPRESENTATIVE DISINFECTANTS ON SOME OF THE MORE IMPORTANT PATHOGENIC BACTERIA.

| Kind of bacteria. | Thermal death-point. | | | Kind of media. | Mercuric chloride, 1 to 1000. | Hydrogen peroxide, 1 to 200. | Carbolic acid, 1 to 100. | Formalin, 1 to 50. | Trichloride of of iodine, 1 to 750. | Copper sulphate, 1 to 200. | Tricresol, 1 to 200. | Chloramin, 1 to 10,000. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 100° | 80° | 55° C | | | | | | | | | |
| Typhoid bacilli | .. | .. | 15[1] | Non-albuminous | 1[1] | 5 | 5 | 5 | .. | .. | 1 | |
| | | | | Albuminous[2] | 5 | 20 | 15 | 15 | .. | .. | 5 | 5 |
| B. coli | .. | .. | 20 | Non-albuminous | 5 | .. | 15 | | | | | |
| | | | | Albuminous | 70 | .. | 70 | 20 | | | | |
| Diphtheria bacillus | .. | 5 | 20 | Non-albuminous | 1 | 10 | 5 | 3 | 1 | 2 | 1 | |
| | | | | Albuminous | 5 | 20 | 10 | 5 | 5 | 5 | 5 | 5 |
| Staphylococcus | 2 | 5 | 60 | Non-albuminous | 15 | 15 | 20 | 20 | | | | |
| | | | | Albuminous | 30 | 30 | 30 | 40 | | | | |
| Streptococcus | .. | .. | 20 | Non-albuminous | 5 | 5 | 5 | 5 | 5 | 5 | 5 | |
| | | | | Albuminous | 10 | 15 | 40 | 30 | .. | 10 | 10 | |
| Pneumococcus | .. | .. | 20 | Non-albuminous | 5 | 5 | 5 | 5 | 5 | 5 | 5 | |
| | | | | Albuminous | .. | .. | 10 | 10 | | | | |
| Meningococcus | .. | .. | 25 | Non-albuminous | 1 | 3 | 1 | 1 | .. | .. | 1 | |
| | | | | Albuminous | 5 | 15 | 5 | 5 | .. | .. | 5 | 5 |
| B. anthracis spores | 150 | .. | .. | Non-albuminous | 180 at 75° C. | .. | 150 at 75° C. | | | | | |
| | | | | Albuminous | | | | | | | | |

[1] The time is given in minutes.

[2] The tests made with albuminous media were by Willa Noble in our Laboratory. The cultures were grown twenty-four hours at 37° C. in glucose ascitic broth, one-tenth of culture inoculated into 5 c.c. of disinfectant, temperature 20° C.

Thymol and eucalyptol have about one-fourth the strength of carbolic acid (Behring).

Oil of peppermint in 1 to 100 solution prevents the growth of bacteria.

TABLES OF ANTISEPTIC VALUES.[1]

| | | | |
|---|---|---|---|
| Alum | 1 to 222 | Mercuric chloride | 1 to 14,300 |
| Aluminum acetate | 1 to 6000 | Mercuric iodide | 1 to 40,000 |
| Ammonium chloride | 1 to 9 | Potassium bromide | 1 to 10 |
| Boric acid | 1 to 143 | Potassium iodide | 1 to 10 |
| Calcium chloride | 1 to 25 | Potassium permanganate | 1 to 300 |
| Calcium hypochlorite | 1 to 1000 | Pure formaldehyde | 1 to 25,000 |
| Carbolic acid | 1 to 333 | Quinine sulphate | 1 to 800 |
| Chloral hydrate | 1 to 107 | Silver nitrate | 1 to 12,500 |
| Cupric sulphate | 1 to 2000 | Sodium borate | 1 to 14 |
| Ferrous sulphate | 1 to 200 | Sodium chloride | 1 to 6 |
| Formaldehyde (40 per cent.) | 1 to 10,000 | Zinc chloride | 1 to 500 |
| Hydrogen peroxide | 1 to 20,000 | Zinc sulphate | 1 to 20 |

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[1] These figures are approximately correct, and represent the percentage of disinfection required to be added to a fluid containing considerable organic material, in order permanently to prevent any bacterial growth. Solutions of half the given strength will inhibit the growth of most bacteria and prevent the growth of many varieties.

# CHAPTER LIII.

# PRACTICAL DISINFECTION AND STERILIZATION (HOUSE, PERSON, INSTRUMENTS, AND FOOD). STERILIZATION OF MILK FOR FEEDING INFANTS.

## DISINFECTANTS AND METHODS OF DISINFECTION EMPLOYED IN THE HOUSE AND SICK-ROOM.

**Disinfection and Disinfectants.**—Sunlight, pure air and cleanliness are always very important agents in maintaining health and in protecting the body against many forms of illness. When, however, it becomes necessary to guard against such special dangers as infectious material from communicable diseases the additional protection of disinfection should be considered. Practical disinfection never affords complete protection; and perfect cleanliness is better, even in the presence of contagious disease, than filth with disinfection, as it is ordinarily carried out. If it is possible to disinfect thoroughly the discharge of patients and of those in contact with them aërial disinfection is unnecessary.

In order that as few articles as possible shall be exposed to the pathogenic germs in the discharges of patients, it is important when conditions allow of it that all articles not necessary for the care and comfort of the sick person, especially upholstered furniture, carpets, and curtains, should be removed from the room before placing the sick person in it.

**Agents for Cleansing and Disinfection.**—Too much emphasis cannot be placed upon the importance of cleanliness, both as regards the person and the dwelling, in protecting the body from all kinds of infectious disease. Personal cleanliness should be attained by frequently washing the hands and body, replacing fabrics infected by expectoration, bowel discharges, etc. By these means most of the pathogenic bacteria are removed before they have caused infection.

Cleanliness in dwellings, and in all places where men go, may, under ordinary circumstances, be well maintained by the use of the two following solutions:

1. **Soapsuds Solution.**—For simple cleansing, or for cleansing after the method of disinfection by chemicals described below, one ounce of common washing soda should be added to twelve quarts of hot soapsuds (soft soap and water).

2. **Strong Soda Solution.**—This, which is a stronger and more effective cleansing solution and also a fairly efficient disinfectant, is made by dissolving one-half pound of common washing soda in three gallons of hot water. The solution thus obtained should be applied by scrubbing with a hard brush.

When it becomes necessary to prevent the spread of communicable diseases by surely killing the living germs which cause them, more powerful agents must be employed than those required for simple cleanliness, and those are commonly called disinfectants. The following are some of the most reliable ones:

1. **Heat.**—Complete destruction by fire is an absolutely safe method of disposing of infected articles of small value, but continued high temperatures not as great as that of fire will destroy all forms of life; thus, boiling or steaming in closed vessels for ten minutes will absolutely destroy all disease germs except spores.

2. **Carbolic Acid Solution.**—Dissolve six ounces of carbolic acid in one gallon of hot water (200 grams in 4000 c.c.). This makes approximately a 5 per cent. solution of carbolic acid, which, for most purposes, may be diluted with an equal quantity of water. The commercial "soluble crude carbolic acid" which is cheaper and twice as effective as the carbolic acid, can be used for privies and drains.[1] It makes a white emulsion on account of its not entering readily into solution. Care must be taken that the pure acid does not come in contact with the skin.

3. **Bichloride Solution** (bichloride of mercury or corrosive sublimate).—Dissolve sixty grains of pulverized corrosive sublimate and two tablespoonfuls of common salt in one gallon of hot water. This solution, which is approximately 1 to 1000, must be kept in glass, earthen, or wooden vessels (not in metal vessels). For safety it is well to color the solution.

The carbolic and bichloride solutions are very poisonous when taken by the mouth, but are harmless when used externally.

4. **Milk of Lime.**—This mixture is made by adding one quart of dry, freshly slaked lime to four or five quarts of water. (Lime is slaked by pouring a small quantity of water on a lump of quicklime. The lime becomes hot, crumbles, and as the slaking is completed a white powder results. The powder is used to make milk of lime.) Air-slaked lime (the carbonate) has no value as a disinfectant.

5. **Dry Chlorinated Lime, "Chloride of Lime."**—This must be fresh and kept in closed vessels or packages. It should have the strong, pungent odor of chlorine.

6. **Formalin** (this is a watery solution containing 40 per cent. of formaldehyde).—Add 1 part of formalin to 10 of water. This equals in value the 5 per cent. carbolic acid solution.

7. **Creolin, Tricresol, and Lysol.**—The first is of about the same value as pure carbolic acid, the latter two about three times as powerful.

The proprietary disinfectants, which are so often widely advertised and whose composition is kept secret, are relatively expensive and often unreliable and inefficient. It is important to remember that substances which destroy or disguise bad odors are not necessarily disinfectants, and that there are very few disinfectants that are not poisonous when taken internally. Their value should be stated in the circular in comparison with pure carbolic acid, so that their strength may be known.

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[1] The cost of the pure carbolic acid solution is much greater than that of most of the other solutions, but except for the disinfection of the skin, which in some persons it irritates, and of woodwork, it is generally much to be preferred by those not thoroughly familiar with disinfectants, as it does not deteriorate, and is rather more uniform in its action than some of the other disinfectants.

**Methods of Disinfection in Infectious and Contagious Diseases.**—The diseases most commonly guarded against, outside of surgery, by disinfection are scarlet fever, measles, diphtheria, tuberculosis, smallpox, typhoid, bubonic plague, and cholera.

1. **Hands and Person.**—Dilute the 5 per cent. carbolic solution with an equal amount of water or use the 1 to 1000 bichloride solution without dilution. Hands soiled in caring for persons suffering from contagious diseases, or soiled portions of the patient's body, should be immediately and thoroughly soaked with one of these solutions and then washed with soap and water, and finally immersed again in the solution. The nails should always be kept clean. Before eating, the hands should be first washed in one of the above solutions, and then thoroughly scrubbed with soap and water by means of a brush.

2. **Soiled Clothing, Towels, Napkins, Bedding, etc.,** should on removal be immediately immersed in the 2.5 per cent. carbolic solution, in the sick-room, and soaked for one or more hours. Articles such as beds, woollen clothing, etc., which cannot be washed, should be thoroughly exposed to formaldehyde gas, as noted later. This is not necessary for goods after exposure to measles.

3. **Food and Drink.**—Food thoroughly cooked and drinks that have been boiled are free from disease germs. Food and drinks, after cooking or boiling, if not immediately used, should be placed when cool in clean dishes or vessels and covered. In the presence of an epidemic of cholera or typhoid fever, milk and water used for drinking, cooking, washing dishes, etc., should be boiled before using, and all persons should avoid eating uncooked fruit and fresh vegetables.

4. **Discharges of all kinds from the Mouth, Nose, Bladder, and Bowels** of patients suffering from contagious diseases should be received into glass, metal, or earthen vessels containing the carbolic solution, or milk of lime, or they should be removed on pieces of cloth, which are immediately immersed in one of these solutions or boiled or destroyed by fire. Special care should be observed to disinfect at once the vomited matter and the intestinal discharges from cholera patients. In typhoid fever the urine and the intestinal discharges, and in diphtheria, measles, and scarlet fever the discharges from the throat and nose all carry infection and should be treated in the same manner. The volume of the solution used to disinfect discharges should be at least twice as great as that of the discharge, and should completely mix with it and cover it. After standing for an hour or more the discharges with the exception of the feces may be thrown into the water-closet.

Masses of feces are extremely difficult to disinfect except on the surface, for it takes disinfectants such as the carbolic acid solution some twelve hours to penetrate to their interior. If fecal masses are to be thrown into places where the disinfectant solution coverin will be washed off, it will be necessary to be certain that the disin has previously penetrated to all portions and destroyed the germs. This can be brought about by stirring them with the fectant and allowing the mixture to stand for one hour, or by w them into a pot holding soda solution which is already at the temperature, or later will be brought to it.

5. **Sputum from Consumptives.**—The importance of the prop infection of the sputum is still underestimated. Consumption infectious disease and is always the result of transmission fr sick to the healthy or from animals to man. The sputum conta germs which cause the disease, and in a large proportion of cases source of infection. After being discharged, unless properly di of, it may become dry and pulverized and float in the air as dust. dust contains the germs, and is a common cause of the disease, tl inhalation. In all cases, therefore, the sputum should be disi when discharged. It should be received in covered cups con the carbolic or milk-of-lime solution. Handkerchiefs soiled by it be soaked in the carbolic solution and then boiled. Dust fr walls, mouldings, pictures, etc., in rooms that have been occup consumptive patients, where the rules of cleanliness have no carried out, contain the germs and will produce tuberculosis in a when used for their inoculation; therefore rooms should be thor renovated or disinfected before they are again occupied. If the s of all consumptive patients were destroyed at once when discha large proportion of the cases of the disease would be prevented.

6. **Closets, Kitchen and Hallways, Sinks, etc.**—The closets should be used for infected discharges until they have been thorough infected; when done, one quart of carbolic solution or of 5 pe solution of formalin should be poured into the pan (after it is em and allowed to remain there. Sinks should be flushed with one o solutions at least once daily.

7. **Dishes, Knives, Forks, Spoons, etc.**, used by a patient shoul rule, be kept for his exclusive use and not removed from the They should be washed first in the carbolic solution, then in l hot soapsuds, and finally rinsed in hot water. The remains patient's meals may be burned or thrown into a vessel containi carbolic solution or milk of lime, and allowed to stand for on before being thrown away.

8. **Rooms and Their Contents.**—When the patient is freed from tion probably the disease germs have already died, but a few ma survived. The danger from infection is much greater when cas removed during the acute illness. For disinfecting rooms careful gation with formaldehyde gas should be employed unless vermin be killed, when sulphur fumes should be substituted. Carpets, cu and upholstered furniture which have been soiled by discharg which have been exposed to infection in the room during the illnes be removed for disinfection to chambers where they can be expo

formaldehyde gas and moderate warmth for twelve to twenty-four hours, or to steam. Some carpets, such as many Wiltons, are discolored by moist steam. These must be put in the formaldehyde chamber. This difficult procedure may be safely omitted, for even if a few germs remain alive in the depth of the fabrics, they probably cannot escape. Wood-work, floors, and plain furniture will be thoroughly washed with soapsuds and bichloride solutions. After the disinfection is finished it is well to remove the dried bichloride of mercury from the walls.

9. **Rags, Cloths, and Articles of Small Value,** which have been soiled by discharges or infected in other ways, should be boiled or burned.

10. **In Case of Death** the body should be completely wrapped in several thicknesses of cloth wrung out of the carbolic or bichloride solution, and when possible placed in a hermetically sealed coffin.

It is important to remember that *an abundance of fresh air, sunlight, and absolute cleanliness* not only helps protect the attendants from infection and aids in the recovery of the sick, but directly eliminate the bacteria which cause disease.

**Methods of Cleanliness and Disinfection to Prevent the Occurrence of Illness.**—1. **Water-closet Bowls and all Receptacles for Human Excrement** should be kept perfectly clean by frequent flushing with a large quantity of water, and as often as necessary disinfected with the crude carbolic, or other efficient solutions. The wood-work around and beneath them should be frequently scrubbed with the hot soapsuds solution.

2. **Cesspools and Privy Vaults.**—An abundance of milk of lime or chloride of lime should be thrown into these daily, and their contents should be frequently removed.

3. **The Wood-work in School-houses** should be scrubbed daily with hot soapsuds. This refers to floors, doors, door-handles, and all wood-work touched by the scholars' hands.

4. **Spittoons in all Public Places** should be emptied daily and washed with the hot soapsuds solution, after which a small quantity of the carbolic solution or milk of lime should be put in the vessel to receive the expectoration. This prevents flies conveying infection.

5. **Cars, Ferry-boats, and Public Conveyances.**—The floors, door-handles, railings, and all parts touched by the hands of passengers should be washed frequently with the hot soapsuds solution. Slat-mats from cars, etc., should be cleaned by scrubbing with a stiff brush in the hot soapsuds solution.

Telephone receiver mouth-pieces should also be frequently cleansed.

**The Practical Employment of Formaldehyde Gas in the Surface Disinfection of Rooms and the Disinfection of Goods which would be Injured by Heat.**—Formaldehyde gas has come into such general use, and is for many purposes so valuable, that the description of methods employed to generate and use it will be given in detail.

If we consider now the practical application of formaldehyde gas for purposes of disinfection we find that its destructive action on micro-organisms depends upon a number of factors, the chief of which are its concentration in the surrounding atmosphere, the length of the contact, the existing temperature, the accompanying moisture, and the of the organism. It is not an insecticide like sulphur dioxide.

**Concentration.**—The necessary concentration of gas in the surroı atmosphere to kill the microörganisms varies with each speci some resist chemical agents much more than others, and also wi freedom of access of the gas to the bacteria, for if they are under or within fabrics a greater amount of gas must be generated t they are freely exposed.

For purely surface disinfection, when the common pathogenic b are to be destroyed, there will be required, according to the n used, ten to twelve ounces of formalin of full strength, or its equi to 1000 cubic feet of air space.

The gas penetrates through fabrics with difficulty, and to pass tl heavy goods the concentration of the gas must be doubled and mo heat added (45° C. or above).

Although formaldehyde gas does destroy bacteria with the a of moisture usually present in the air, it acts much more pow and certainly when additional moisture is present, and best present up to the point of saturation. The actual spraying with of walls and goods to be disinfected is even more efficacious.

**Temperature.**—A fairly high temperature—but one still belov which would injure delicate fabrics—increases not only the acti formaldehyde gas, but also its penetrative power, and for heavy it is essential. The production of a partial vacuum in the cha before the introduction of the formaldehyde gas still further assi penetration.

**Length of Exposure.**—This depends upon the nature of the for which it is carried out—the penetration required, the concent of the gas used, the amount of moisture in the air, the temperat the air, and the size and shape of the room. For surface disin in rooms, when as much as twelve ounces of formalin are used fo 1000 cubic feet, five hours' exposure is amply sufficient, most b being killed within the first thirty minutes. For the destruct microörganisms protected by even a layer of thin covering, the formalin and double the time of exposure should be allowe even then the killing of many species of non-sporebearing b cannot be counted upon in ordinary rooms. When absolutely co disinfection is demanded, where penetration of gas is require goods must be placed in chambers where moderate heat can be and all leakage of gas prevented.

In order to insure complete sterilization of the articles they be so placed as to allow of a free circulation of the gas around —that is, in the case of bedding, clothing, etc., these should be spread out on perforated wire shelves or loosely suspended chamber. The aid of a partial vacuum facilitates the operation holstered furniture and articles requiring much space should be in a large chamber, or, better, in a room which can be heated required temperature.

The most delicate fabrics, furs, leather, and other articles, which are injured by steam, hot air at 230° F., or other disinfectants, are unaffected by formaldehyde.

DISINFECTION OF BOOKS.—Books may be satisfactorily disinfected by means of formaldehyde gas in a special room, or in the ordinary steam chamber, as above described, and under the same condition of volume of gas, temperature, and time of exposure. The books should be arranged to stand as widely open as possible upon perforated wire shelves, set about one or one and a half feet apart in the chamber. A chamber having a capacity of 200 to 250 cubic feet would thus afford accommodation for about one hundred books at a time.

Books, with the exception of their surfaces, cannot be satisfactorily disinfected by formaldehyde gas in the bookcases of houses or libraries, or anywhere except in special chambers constructed for the purpose, because the conditions required for their thorough disinfection cannot otherwise be complied with.

DISINFECTION OF CARRIAGES, ETC.—Carriages, ambulances, cars, etc., can easily be disinfected by having built a small, tight building, in which they are enclosed and surrounded with formaldehyde gas. Such a building is used for disinfecting ambulances in New York City. With the apparatus there employed a larger amount of formalin is rapidly vaporized, and superficial disinfection is completed in sixty minutes.

**Methods of Generating Formaldehyde Gas.**—Various forms of apparatus can be properly employed to liberate formaldehyde gas for purposes of disinfection. There are two essentials to any good method—namely, that the formaldehyde gas is given off quickly, and that there is no great loss by deterioration of the formalin.

**Wood Alcohol.**—A number of lamps have been devised, all very much on the same principle, though varying somewhat in mechanical construction, which bring about the incomplete oxidation of methyl alcohol by passing the vapors mixed with air over the incandescent metal. Although disinfection can be carried out by the best of these lamps, in our experience none of them up to the present time is satisfactory or economical. They may be very useful as deodorizers in the sick-room or other places.

The same principle is used efficiently in another form. The vapor of wood alcohol is passed over the surfaces of asbestos containing particles of finely divided platinum. This apparatus has given very good results, and for a given amount of disinfection leaves less odor of formaldehyde gas in the room than any other. The apparatus is, however, bulky and expensive.

**Formalin by Boiling and Passing the Vapor through a Superheated Coil or Chamber.**—This system consists in heating the ordinary commercial formalin to a high temperature in an incandescent copper coil or chamber, and allowing the vapors to pass off freely. It is claimed for this method that the degree of heat necessary to break up the polymerized products formed is supplied, and thus a loss of formaldehyde is prevented. A further action of the intense heat in the copper tube on the solution is partially to convert the methyl alcohol contained in commercial formalin into formaldehyde gas by partial oxidation, thereby utilizing a part of the methyl alcohol and increasing the amount of formaldehyde (Fig. 213).

**Trioxymethylene or Paraform.**—This system co
solid polymer of formaldehyde (trioxymethylene).
There are several methods for doing this.
expensive but efficient. The formaldehyde gas
from burning.

**Formalin to which Glycerin has been Added.**—To
10 per cent. of glycerin, and the mixture is simply boil
vessel, the steam and formaldehyde gas passing off by
serviceable apparatus. When it is attempted to vap
rapidly part of it bubbles over in fluid form.

With 50 per cent. more of formalin than that used i
autoclave and heated tube or chamber methods, the res
as good. The apparatus is very easy to use, and is not li

Similar forms of apparatus are also employed, whe
the formalin is mixed with an equal quantity of water.
purpose of giving additional moisture to the air, and, at
glycerin, to prevent the change of formaldehyde into in

**From Formalin in an Open Pan.**—A very simple m
R. J. Wilson, is to fill a pan with 12 ounces of formalin
and put this on an upright sheet of tin, which is cut so as
of air below and yet protect the formalin in the pan fro
ing put under it a small tin can filled with asbestos
soaked with wood alcohol. A still simpler method is t
16 ounces of formalin per 1000 cubic feet and then stre
in a room and leave for two hours. If the room is
surface disinfection will take place.

**Lime and Permanganate Method of Generating**
Satisfactory results in disinfection have been obtain
combination of chemicals. Two ounces of a quick-sla
lime (calcium oxide); 5 ounces of permanganate of pot
5 ounces formaldehyde solution, 40 per cent. strength;
This is sufficient in quantity to disinfect 1000 cubic feet
It is used as follows: The lime and permanganate of po
in a pan at least 10½ inches in diameter and 3½ to 4
this is poured the freshly prepared mixture of formal
acid and water. A rapid evolution of gas takes place.

Another combination is: lime, 2.7 ounces; potassi
ounces; formaldehyde solution, 7.4 ounces; water, 2.7
is as follows: The lime and permanganate are mixed
above, and the freshly prepared formaldehyde and w
over it.

Permanganate of potash method. The following com
fect 1000 cubic feet of space in five hours: potassium pe
formaldehyde solution, 40 per cent., 9 ounces; water,
maldehyde and water are mixed together and rapidly p
ganate of potash. The reaction is immediate and
requires a deep, wide pan or a pail at least 18 inches dee
water is believed to increase the liberation of the formal

**Lime Method of Generating Formaldehyde Gas.**—T
cent. formaldehyde solution slowly add 1 ounce of
acid; pour this solution into 2 pounds of quicklime th
cracked into small lumps and placed in a dairy pan no
diameter. The liberation of a large amount of gas in
compensates for the loss of polymerization, and disinf
quick union of the gas and organisms to be destroyed.
aluminum sulphate may be used instead of concentrat

**Rapid Generation of Formaldehyde Gas for Large Chambers by the Method of Dr. R. J. Wilson.**—The generator (Fig. 214) is made of ordinary iron steam pipe and can be manufactured in any pipe-cutting establishment in a very few hours. It consists of an outer steam jacket of six-inch pipe, two feet long and capped at both ends. Through the upper cap there is a four-inch opening with a thread, through which projects an inner chamber for formalin. This chamber consists of a four-inch pipe, twenty-two inches long, capped at the upper end and welded or capped at the lower end. The upper end of this pipe is so threaded as to permit of its being screwed through the cap of the steam jacket before that cap is screwed on. The cap of the formalin chamber is fitted on the same thread that passes through the cap of the steam jacket. The intake for steam is near the top of the steam jacket, through a half-inch pipe, and the steam is controlled by a globe valve. The outlet for steam or drip is through a half-inch pipe from the bottom cap of the chamber and is also controlled by a globe valve. The intake for formalin is through the upper cap of the formalin chamber through a half-inch pipe controlled by a globe valve. The outlet for formaldehyde is a half-inch pipe through the upper cap of the formalin chamber.

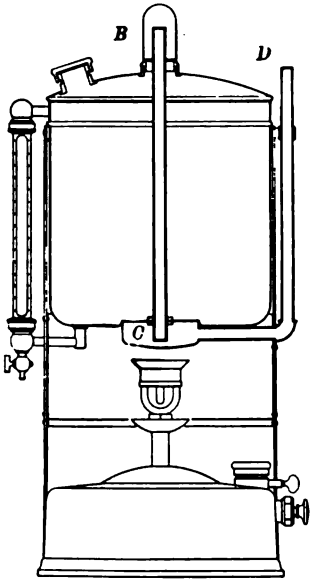

FIG. 213.—Formaldehyde apparatus.

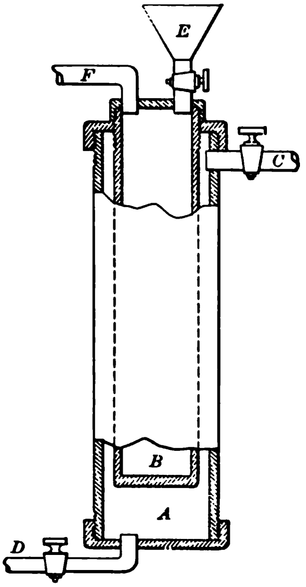

FIG. 214.—Wilson's formaldehyde generator: *A*, steam chamber; *B*, formalin chamber; *C*, steam supply; *D*, drip; *E*, inlet for formalin; *F*, outlet for formaldehyde.

As a result of the investigations undertaken in the Department of Health laboratories on methods for testing the efficacy of room disinfections, we adopted the plan of placing infected threads in the rooms. This plan adopted by us may be summarized as follows:

METHOD FOR TESTING EFFICACY OF ROOM DISINFECTION.—The following method which was modified by Dr. Schroder, working in the Research Laboratory, has been used in the Department of Health, The main points of the system are as follows:

No. 36 cotton is cut into inch lengths, placed in a Petri dish, and covered with a forty-eight-hour broth culture of B. pyocyaneus.

They are left for two or three minutes or until they are thoroughly saturated, then removed to filter-paper in another covered Petri dish and left to dry. When dry they are placed in tissue-paper envelopes, which are stamped with all necessary data. Each envelope is dated and sealed and sent to the disinfector who places it in the room which is to be disinfected.

The driver who calls for the bedding takes up the tests, placing them in a manila envelope and entering them upon his card. The envelopes are then returned to the laboratory where the tests and receipt card are compared and any discrepancy noted.

The test envelopes are then stamped with date of receipt. and the threads are removed and placed in a modified Ayer's medium, which is a synthetic medium and consists of the following:

| | |
|---|---|
| Asparagin | 4 |
| Neutral sodium phosphate | 2 |
| Sodium lactate | 6 |
| Sodium chlorate | 5 |
| Water | 1000 |

Add enough NaOH to render the medium alkaline to litmus. This culture medium may be depended upon to give bright green color reaction in twenty-four to forty-eight hours.

The tubes are incubated for forty-eight hours and the color reaction noted and entered upon test envelope.

At the end of the week a bacteriologist's report is compiled which shows at a glance the work of each disinfector, the number of cases of each disease for which disinfection was performed, the number of successful disinfections, the number of tests lost, etc.

**Sulphur Dioxide in House Disinfection.**—Four pounds of sulphur should be burned for every 1000 cubic feet. The sulphur should be broken into small pieces and put into a pan sufficiently large not to allow the melted sulphur to overflow. This pan is placed in a much larger pan holding a little water. The cracks of the room should be carefully pasted up and the door, after closing, also sealed. Upon the broken sulphur is poured three or four ounces of alcohol and the whole lighted by a match. The alcohol is not only for the purpose of aiding the sulphur to ignite, but also to add moisture to the air. An exposure of eight to twelve hours should be given.

Sulphur fumigation carried out as above indicated is not as efficient as formaldehyde fumigation, but suffices for surface disinfection for diphtheria and the exanthemata. All heavy goods should be removed for steam disinfection if there is any possibility of the infection having penetrated beneath their surface. If there is no place for steam disinfection their surfaces should be thoroughly exposed to fumigation and then to the air and sunlight. In many cases when cleanliness has been observed, surface disinfection of halls, bedding, and furniture may be all that will be required.

There is always a very slight possibility of a deeper penetration of infection than that believed to have occurred; it is therefore better to be more thorough than is considered necessary rather than less.

Sulphur dioxide without the addition of moisture has, as already stated under the consideration of disinfectants, very little germicidal value upon dry bacteria.

ADVANTAGES OF FORMALDEHYDE GAS OVER SULPHUR DIOXIDE FOR DISINFECTION OF DWELLINGS.—Formaldehyde gas is superior to sulphur dioxide as a disinfectant for dwellings; first, because it is more efficient in its action; second, because it is less injurious in its effects on household goods; third, because when necessary it can easily be supplied from a generator placed outside of the room and watched by an attendant, thus avoiding, in some cases, danger of fire.

Apart from the cost of the apparatus and the greater time involved, formaldehyde gas, generated from commercial formalin, is not much more expensive than sulphur dioxide—viz., twelve to twenty cents per 1000 cubic feet against ten cents with sulphur. Therefore, we believe that formaldehyde gas is the best disinfectant at present known for the surface disinfection of infected dwellings. For heavy goods it is far inferior in penetrative power to steam; but for the disinfection of fine wearing apparel, furs, leather, upholstery, books, and the like, which are injured by great heat, it is, when properly employed, better adapted than any other disinfectant now in use.

**Public Steam Disinfecting Chambers.**—These should be of sufficient size to receive all necessary goods, and may be either cylindrical or rectangular in shape, and are provided with steam-tight doors opening at either end, so that the goods put in at one door may be removed at the other. When large the doors are handled by convenient cranes and drawn tight by drop-forged steel eye-bolts swinging in and out of slots in the door frames. The chambers should be able to withstand a steam pressure of at least one-half an atmosphere, and should be constructed with an inside jacket, either in the form of an inner and outer shell or of a coil of pipes. This jacket is filled with steam during the entire operation, and is so used as to bring the goods in the disinfecting chamber up to the neighborhood of 220° F. before allowing the steam to pass in. This heats the goods, so that the steam does not condense on coming in contact with them. It is an advantage to displace the air in the chamber before throwing in the steam, as hot air has far less germicidal value than steam of the same temperature. To do this a vacuum pump is attached to the piping, whereby a vacuum of fifteen inches can be obtained in the chamber. The steam should be thrown into the chamber in large amount, both above and below the goods, and the excess should escape through an opening in the bottom of the chamber, so as more readily to carry off with it any air still remaining. The live steam in the chamber should be under a pressure of two or three pounds so as to increase its action.

To disinfect the goods we place them in the chamber, close tight the doors, and turn the steam into the jacket. After about ten minutes, when the goods have become heated, a vacuum of ten to fifteen inches is produced, and then the live steam is thrown in for twenty minutes. The steam is now turned off, a vacuum is again formed, and the chamber again superheated. The goods are now thoroughly disinfected and dry. In order to test the thoroughness of any disinfection, or any new chamber, maximum thermometers are placed, some free in the chamber and others surrounded by the heaviest goods. It will be found that, even under a pressure of three pounds, live steam will require ten minutes to penetrate heavy goods.

**Practical Points on Heat Disinfection.**—In the practical application of steam for disinfecting purposes it must be remembered that while moist steam under pressure is more effective than streaming steam, it is scarcely necessary to give it the preference, in view of the fact that most known pathogenic bacteria produce no spores and the spores of the few that do develop them are quickly destroyed by the temperature of boiling water, and also that "superheated" steam is less effective than moist steam. When confined steam in pipes is "superheated" after its generation it has about the same germicidal power as hot, dry air at the same temperature. Esmarch found that anthrax spores were killed in streaming steam in four minutes, but were not killed in the same time by superheated steam at a temperature of 114° C. It should also be remembered that dry heat has but little penetrating power, and that even steam requires time to pass through heavy goods. Koch and Wolffhügel found that registering thermometers placed in the interior of folded blankets and of other large packages did not show a temperature capable of killing bacteria after three hours' exposure in a dry hot-air oven at 133° C. and over. We have put a piece of ice in the middle of several mattresses and recovered it after exposing the goods to an atmosphere of live steam for ten minutes.

**The Disinfection of Instruments, Ligatures and Dressings for Surgical Operations.**—**Instruments.**—All instruments, except knives, after having been thoroughly cleansed, are boiled for three minutes in a 1 per cent. solution of washing soda. Knives, after having been thoroughly cleansed, are washed in sterile alcohol and wiped with sterile gauze and then put into boiling soda solution for one minute This will not injure their edges to any great extent.

**Gauze.**—Gauze is sterilized by moist heat either in an Arnold steam sterilizer for one hour or in an autoclave for thirty minutes. It is placed in a perforated cylinder or wrapped in clean towels before putting in the sterilizer, and only opened at the operation.

Iodoform gauze is best made by sprinkling sterile iodoform on plain gauze sterilized as described above.

**Ligatures—Catgut.**—Boil for one hour in alcohol under pressure at about 97° C. It is often put in sealed glass tubes, which are boiled under pressure. These remain indefinitely sterile. The alcohol does not injure the catgut. If desired, the catgut can be washed in ether and then soaked a short time in bichloride before heating in alcohol. Böckman, of St. Paul, suggested wrapping the separate strands of catgut in paraffin paper and then heating for three hours at 140° C. This procedure prevents the drying out of the moisture and fat from the catgut, so that it remains unshrivelled and flexible after its exposure. Darling, of Boston, tested this method and found it satisfactory. Dry formaldehyde gas does not penetrate sufficiently, and is not reliable. Silver wire, silk, silkworm gut, rubber tubing, and catheters are boiled the same as the instruments.

**Hand-brushes.**—These should be boiled in soda solution for ten minutes.

**The Skin of the Patient.**—It is impossible absolutely to sterilize the deeper portions of the skin, but sufficient bacteria can be removed to render infection rare. The skin is washed thoroughly with warm green soap solution, then with alcohol, and finally with 1 to 1000 bichloride. A compress wet with a 25 per cent. solution of green soap is now placed on, covered with rubber tissue, and left for three to twelve hours; and after its removal the skin is washed with ether alcohol, and bichloride solution, and then covered with a gauze compress previously moistened with a 1 to 1000 bichloride of mercury solution. At the operation the skin is again scrubbed with green soap solution followed by ether, alcohol, and then with the bichloride of mercury solution. In some places the bichloride compress is replaced one hour before the operation by a pad wet in 10 per cent. solution of formalin.

**The Hands.**—Fürbinger's method, slightly modified, is now much used, and gives good results. The hands are washed in hot soap and water for five minutes, using the nail-brush. They are then soaked in 85 per cent. alcohol for one minute and scrubbed with a sterile brush. They are finally soaked in a 1 to 1000 bichloride of mercury solution for two minutes. The alcohol and bichloride of mercury are sometimes combined and used together. Another method which gives good results is as follows: Skin of operator is scrubbed for five minutes with green soap and brush, then washed in chorinated lime and carbonate of soda in proportions to make a good lather; washed off in sterile water, and then scrubbed with brush in warm bichloride solution 1 to 1000.

Owing to the risk of leaving untouched bacteria under the nails and in cracks of the skin, sterilized rubber gloves are now being used more and more in operations. Some surgeons prefer sterilized cotton gloves frequently changed. The gloves can be sterilized by steam.

**Mucous Membranes.**—Here absolute sterilization cannot be achieved without serious injury to the tissues. Those of the mouth and throat are cleansed by a solution consisting of equal parts of peroxide of hydrogen and lime-water. In the nostrils it is better to employ the milder solutions, such as diluted Dobell's or Listerine. These are also used in the mouth instead of the peroxide. Wadsworth[1] urges the use of preparations containing about 30 per cent. of alcohol as being very efficient. Dakin's solution (see below) may also be tried.

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[1] Jour. Inf. Dis., 1906, p. 779.

The vagina is swabbed out thoroughly with sterile warm soap and water, and then irrigated with a 2 per cent. carbolic acid or a 1 to 1000 bichloride of mercury solution.

**Disinfection of Wounds.**—The immense number of wounded in the European War has led to a further study of the use of antiseptic solutions.

The great efficiency of chlorine and some of its less stable compounds as purifying, deodorizing and bleaching agents has for a long time been known. Its action was attributed to the strong indirect oxidizing influence exerted by it. Chlorine gas and calcium hypochlorite, "bleaching powder" or "chloride of lime" were recognized as among the most potent, though destructive disinfectants. Potassium and sodium hypochlorite were also employed as disinfectants and to some extent as antiseptics, though the latter use was restricted by the irritating action of these substances.

It was not until the present war in Europe created a demand for more efficient antiseptics than those in general surgical use that recourse was made to chlorine compounds in which the chlorine existed in a combination from which it could be readily liberated in active form according to requirements.

The researches of Dr. H. D. Dakin[1] at first associated with Dr. Alexis Carel in France and subsequently commissioned by the British Government, demonstrated that sodium hypochlorite in strictly neutral solution and not exceeding 0.5 per cent. in concentration, could be used freely in surgical practice without occasioning discomfort.[2] His studies on the action of this antiseptic showed that when it acted on protein substances it conferred antiseptic properties on them and that the chlorine presumably became linked to a nitrogen of one or more of the amino acids composing the protein. This led to the inference that soluble substances possessing a nitrogen-chlorine linkage would be likely to have antiseptic properties. This was verified by experiment. The group of substances, called chloramines, which have this linkage, contain many preparations which are soluble and very slightly, if at all, toxic. The cheapest of these, because it can be manufactured from a waste substance obtained in the production of saccharin, is paratoluenesodiumsulphochloramide, $C_6H_5SO_2NaNCl.3H_2O$. This substance is obtained in white crystals, readily soluble in water and singularly stable both dry and when in solution. It readily parts with chlorine when brought into contact with easily oxidizable substances, and is a powerful antiseptic. Proteins are not precipitated by this chloramine, and its penetration when applied to the tissues is unusually great. As it is decomposed in exerting its action, it should be frequently renewed, but its non-toxic and unirritating characters permit its free and abundant use. Besides Dakin's solution other antiseptic irrigation fluids are being experimented with in the treatment of the wounded, and also ointments and powders. It is believed that for certain infections special antiseptics should be selected. In the latter part of the war fresh wounds were usually closed without the use of antiseptics when the injured tissues could be first removed.

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[1] British Med. Jour., 1915, ii, 318; Ibid., January 29, 1916, p. 160.

[2] A neutral sodium hypochlorite solution of proper strength, "Dakin's solution," can be prepared as follows: Dissolve 14 grams of dry sodium carbonate (or the equivalent of the hydrated crystals) in 1 liter of water, add 20 grams of fresh bleaching powder with not less than 30 per cent. available chlorine and shake the mixture vigorously for at least five minutes. Allow it to stand for half an hour and then filter. To the clear filtrate, add 2 grams of boric acid, which should so completely neutralise the solution that a particle of solid phenolphthalein will not become pink when moistened with it. It is also possible to prepare a neutral solution of sodium hypochlorite without the addition of boric acid, if sodium bicarbonate is employed. With the bleaching powder of the quality mentioned, the proportions may be chosen as follows: 45 grams dry sodium carbonate and 45 grams of sodium bicarbonate are dissolved in 1 liter of water. One hundred grams of bleaching powder are well shaken with 1 liter of water and then the alkaline solution added and the whole again thoroughly shaken. The precipitate is then removed by filtration. The resulting clear solution should react neutral to phenolphthalein. Should this not be the case, a little carbon dioxide may be passed into the solution to neutralise the residual trace of acid. This solution is three times the strength which should be used. When required, it should be diluted with two volumes of water. Sodium hypochlorite solutions deteriorate. They should not be used when over a week old.

**Hypodermic and Other Syringes.**—These when not boiled are sterilized by drawing up into them boiling water a number of times and then finally a 5 per cent. solution of carbolic acid, the acid after three minutes to be washed out by boiling water. If cold water is used the carbolic solution should remain in the barrel for ten minutes. Great care should be taken to wash out all possible organic matter before using the carbolic acid or boiling to sterilize. Syringes made entirely of glass or of glass and asbestos can be boiled in soda solution.

**The Sterilization of Milk.**—Complete sterilization destroys all the germs in milk, and so, if no new ones gain entrance, prevents permanently fermentative changes. This requires boiling for fifteen to forty-five minutes on two or three consecutive days, according to the presence or absence of certain spores.

Milk is best sterilized by heat, for nearly all chemicals, such as boric acid, salicylic acid, and formalin, are not only slightly deleterious themselves but also make the milk less digestible, and therefore less fit for food. Formalin is the least objectionable of the three. Milk may be sterilized at a high or low temperature—that is, at the boiling temperature—or at a lower degree of heat, obtained by modifying the steaming process. Milk heated at as high a temperature as 100° C. is not altogether desirable for prolonged use for infants, as the high temperature causes certain changes in the milk which make it less suitable as a food for them.

**Pasteurization.**—These changes are almost altogether avoided if a temperature below 70° C. is used. It is recommended, therefore, that the lowest temperature be used for partial sterilization which will keep the milk wholesome for twenty-four hours in the warmest weather and kill the tubercle, typhoid, and other non-sporebearing bacilli. Raising the milk to a temperature of 60° C. for twenty minutes, 65° C. for fifteen, 70° for five, 75° for two, or 80° for one will accomplish this. Exposure for even one minute at 70° destroys 98 per cent. of the bacteria which are not in the spore form. Fully 99 per cent. of tubercle bacilli are destroyed. This subject is considered more fully in the chapter on Milk. One of the many forms of apparatus is the following:

(*a*) A tin pail or pot, about ten inches deep by nine inches in diameter, provided with the ordinary tin cover which has been perforated with eight holes each an inch in diameter.

(*b*) A wire basket, with eight nursing bottles (as sold for this purpose in the shops).

(*c*) Rubber stoppers for bottles and a bristle brush for cleaning the bottles.

**Directions** (Koplik).—Place the milk, pure or diluted (as the physician may direct), in the nursing bottles and place the latter in the wire basket. Put only sufficient milk for one nursing in each bottle. Do not cork the bottles at first.

Having previously poured about two inches of water in the tin pail or pot and brought it to the boiling-point, lower the basket of nursing bottles slowly into the pot. Do not allow the bottles to touch the water or they will crack. Put on the perforated cover and let the steaming continue for ten minutes; then remove the cover and firmly cork each bottle. After replacing the cover, allow the steaming to continue for fifteen minutes. The steam must be allowed to escape freely or the temperature will rise too high.

The process is now complete. Place the basket of bottles in a cool, dark place or in an ice-chest. The bottles must not be opened until just before the milk is to be used, and then it may be warmed by plunging the bottle in warm water. If properly prepared the milk will taste but little like boiled milk.

The temperature attained under the conditions stated above will not exceed in extreme cases 87° C. (188° F.).

A different but admirable method is the one devised by Dr. Freeman. Here a pail is filled to a certain mark with water, and then placed on the stove until the water boils. It is then removed, and immediately a milk-holder, consisting of a series of zinc cylinders, is lowered with its milk bottles partially full of milk. The cover is again applied. The heat of the outside water raises the temperature of the milk in ten minutes to about 65° C. (150° F.), and holds it nearly at that point for some time. After twenty minutes the milk is removed, placed in cold water, and quickly cooled. The milk is kept in the ice-chest until used. When milk is pasteurized in great quantities it should always be done by the "holding process," as "flash" pasteurization is unreliable. Milk should be pasteurized when it is as fresh as possible, and only sufficient milk for twenty-four hours should be pasteurized at one time. If after nursing the infant leaves some milk in the bottle this should be thrown away.

**Care of the Bottles.**—After nursing, the bottles should be filled with a strong solution of washing soda, allowed to stand twenty-four hours, and then carefully cleaned with a bristle (bottle) brush. The rubber stoppers and nipples after using should be boiled in strong soda solution for fifteen minutes and then rinsed and dried.

After sterilizing, milk should never be put into unsterilized bottles, as this will spoil it.

## GENERAL CONCLUSIONS ON DISINFECTION.

The previous pages have shown that it is comparatively easy to destroy microbes by germicides or heat when access to them is attainable. Foods, instruments, clothing, bedding, the excreta, the surface of the body, etc., can be readily disinfected, but when we try to disinfect the mucous membranes of the living person we fail.

*The Importance of Disinfection of Surroundings after Recovery or Death from Infectious Diseases.*—Year by year knowledge is accumulating which indicates that nearly all cases of spread of infection are due to the immediate transfer from a living carrier to the person who contracted the disease or to the indirect transfer through the contamination of food or water. The carrier may be diseased or may simply harbor the germs. If cleanliness is maintained throughout the disease there is, as a rule, little need of specific disinfection after recovery.

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